CHARACTERIZATION OF MITOCHONDRIAL TRANSCRIPTS ASSOCIATED WITH CYTOPLASMIC MALE STERILITY IN *PHASEOLUS VULGARIS* L.

By

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Mitochondrial genes are associated with the CMS trait and are transmitted through the maternal parent. In the common bean, mitochondrial DNA (mtDNA) sequences of 3.7 kb designated *pvs* are associated with CMS. A 7.0 kb RNA is the most abundant among transcripts containing *orf239* of the *pvs*, and has been known to be associated with polyribosomes. A 2.1 kb segment of the 7.0 kb transcript was not accounted for by hybridization with *pvs* and flanking mtDNA clones. In the present study, I have investigated the origin of this 2.1 kb segment and the RNA editing patterns of the *pvs* transcripts.

CMS-unique *atpA/orf209* transcripts (3.0, 2.8, and 2.5 kb) were found by northern analysis in addition to previously identified transcripts. Three C-to-U partial RNA editing events were revealed in the *orf239* coding region of the *pvs* transcripts, whereas three sites were completely edited in the 3' *cob* segment co-transcribed with the *orf239*. The concomitant occurrence of partial and complete editing patterns suggested that transcript abundance does not affect the frequency of RNA editing. The RNA editing events and presence of *orf239*-homologous sequences in mitochondria of other plants suggested that *orf239* could be part of an as-yet-unidentified mitochondrial protein coding gene.

The origin of the CMS-unique 7.0 kb transcript was investigated. Oligonucleotide-directed RNase H digestion assays indicated that the physical structure of the 7.0 kb *pvs* RNA was linear and that the unidentified 2.1 kb segment was located 5' of the *pvs* sequence on this linear molecule. Southern hybridization and PCR analysis revealed a CMS-unique 'sublimon' containing the mitochondrial 18S/5S rRNA sequence, a 3' portion of *orf209* and the *pvs* sequence. RT-PCR analysis verified that this sublimon configuration was transcribed in CMS cytoplasms. The *pvs* sublimon molecule is transcribed to give rise to possibly the 7.0 kb *pvs* RNA.

INTRODUCTION

Mitochondria of higher plants exhibit non-Mendelian inheritance (Corens, 1909) and contain genetic information (Suyama and Bonner, 1966), since the discovery of mitochondria (Meves, 1904). The major functions of the mitochondria are energy production by oxidative phosphorylation, electron transport pathway, and citric acid cycle (reviewed by Tzagoloff, 1982). Although the mitochondria express their own genetic information, nuclear gene products are required for biogenesis and biological functions. The mitochondria of higher plants also have been found to have the genes associated with the cytoplasmic male sterility (CMS) phenotype (reviewed by Hanson and Conde, 1985; Hanson, 1991).

CMS, the failure to produce functional pollen, is the result of mutation in the mitochondrial genome. This trait is inherited through the female parent (Rhoades, 1950). CMS enables the production of F1 hybrid seed by eliminating the need for emasculation. Plant F1 hybrids often result in increased yield. Nuclear fertility restoration genes can suppress or overcome the expression of mitochondrial genes to restore male-fertility (Hanson and

Conde, 1985). The CMS system was exploited as convenient genetic materials for molecular investigations of mitochondrial-nuclear interactions in higher plants.

In *Phaseolus vulgaris*, mtDNA (mtDNA) sequences (3,736 bp) designated *pvs* (for ***Phaseolus vulgaris*** sterility) are associated with CMS (Chase and Ortega, 1992; Johns et al., 1992). At least two different nuclear fertility restoration systems have been identified (Mackenzie et al., 1988; Mackenzie, 1991). The nuclear fertility restorer gene *Fr* irreversibly eliminates the *pvs*-containing mitochondrial DNA (Mackenzie et al., 1988) while another fertility restorer *Fr2* does not alter the mitochondrial genome; the *pvs* sequence is present in the *Fr2* restored line (G08063) (Mackenzie and Chase, 1990; Mackenzie, 1991). As *pvs* is lost upon spontaneous reversion to fertility, the *pvs* region is absent in spontaneous revertant line (WPR-1) and *Fr*-restored lines (Johns et al., 1992; Mackenzie and Chase, 1990; Mackenzie et al., 1988).

Previous northern hybridization analysis revealed CMS-unique *pvs* transcripts of 7.0, 6.8, 4.7, 3.3, and 2.8 kb (Mackenzie and Chase, 1990; Chase, 1994). The 7.0 kb is the most prominent transcript, and sedimentation assays indicate that the 7.0 kb transcript is associated with polyribosomes

(Chase, 1994). A 2.1 kb segment of the 7.0 kb transcript is not accounted for by northern hybridizations with the *pvs* or flanking DNA clones (Chase, 1994). It was suggested that RNA splicing and/or unidentified *pvs* DNA configurations could explain the origin of the unidentified 2.1 kb in the CMS-unique 7.0 kb transcript (Chase, 1994). The first objective of this study was the identification of this unidentified 2.1 kb segment and the characterization of its sequence to better understand the expression of *pvs*-encoded gene products associated with CMS. Identification of the unidentified 2.1 kb segment might potentially reveal novel, CMS-unique open reading frame(s). A DNA configuration containing 18S/5S and the *pvs* sequence was identified at some low level in the male-sterile cytoplasms. This suggests that the molecular heterogeneity of the common bean mitochondrial genome may be generated by recombination at repeated regions (350 bp of *orf209*) in the mtDNA. However, northern analysis could not confirm the hybridization of the 18S/5S probes to the 7.0 kb *pvs* transcript. Further study will be required to show that the *pvs* 7.0 kb resulted from transcription of the CMS-unique sub-stoichiometric mtDNA configuration with the 18S/5S rRNA genes and the *pvs* sequence.

The *pvs* region is flanked by two different repeat sequences X and Y in a configuration designated *config-pvs*. The configurations designated *config-rx* and *config-ry* contain other copies of repeat X or repeat Y, respectively. In the *pvs,* the longest open reading frame is *orf239*, predicting a protein of 239 amino acids and unknown origin. The repeat x includes a complete copy of the F1 ATPase subunit A (*atpA*) gene and an overlapping open reading frame (*orf209*). An apocytochrome b (*cob*) gene segment (117 nt) is located in repeat y. The second objective was to investigate the RNA editing pattern of the *pvs* transcripts to examine the potential of this process to create novel open reading frames. This also addressed the question of the RNA editing pattern of identical sequences in different contexts. This work was approached through the analysis of cDNA clones.

To determine if RNA editing pattern can be altered by sequence context between two configurations with different 3' flanking sequences, cDNA clones from *config-pvs* and *config-rx* covering *atpA*/*orf209* and the *pvs* were examined. Three C-to-U partial RNA editing events were revealed in the *orf239* coding region of the *pvs* transcripts, whereas three sites were completely edited in the 3' *cob* segment co-transcribed with the *orf239*. The concomitant occurrence of partial and complete editing patterns found in the

pvs transcripts suggested that transcript abundance or stability does not affect the frequency of RNA editing for these transcripts. RNA editing events found in *orf239* raise the possibility that the *orf239* could be part of an as-yet-unidentified mitochondrial protein coding gene in plants. Southern analysis of total DNAs with the *orf239* probe revealed hybridizing sequences in tobacco, watermelon, broad-bean, soybean, pea, clover, and peanut.

In addition, I characterized the *atpA/orf209* locus from CMS and revertant cytoplasms in *Phaseolus vulgaris* to gain further information on sequences involved in the CMS phenotype. Previous studies revealed three abundant *atpA/orf209* transcripts (3.0, 2.8, and 2.5 kb) in CMS and revertant lines (Mackenzie and Chase, 1990; Chase, 1994). However, additional *atpA/orf209* transcripts (3.0, 2.8, and 2.5 kb) were found to be unique in CMS-Sprite.

In conclusion, RNA editing studies indicated that the frequency of RNA editing was sequence-specific and was irrelevant to transcription stability. The CMS-unique sublimon configuration containing 18S/5S rRNA genes and the *pvs* was identified and shown to be transcribed possibly giving rise to the *pvs* 7.0 kb transcript.

CHAPTER1: RNA EDITING OF MITOCHONDRIAL TRANSCRIPTS ASSOCIATED WITH CYTOPLASMIC MALE STERILITY IN PHASEOLUS VULGARIS L.

Literature Review

Mitochondrial Genomes in Higher Plants

The mitochondrial genomes of higher plants (reviewed by Fauron, 1995a,b; Pring and Lonsdale, 1985; Bendich, 1993; Schuster and Brennicke, 1994) are not only large in size, but also variable in their organization, compared with their animal or fungal counterparts, as shown in Table 1-1 (reviewed by Shadel and Clayton, 1997; Attardi, 1988). The size of the mitochondrial genome, from 208 kb in *Brassica hirta* (Palmer and Hebron, 1987) to approximately 2,500 kb in muskmelon (Ward et al., 1981), greatly exceeds the amount of

Table 1-1. Mitochondrial genomes in eukaryotes.

	Animal	Fungal	Plant
Genome Size	16 – 17 kb	17 – 115 kb	208 – 2,500 kb
Organization	Compact	Intermediate	Loose
Major form	Circular	Linear	Linear
Gene order	Invariant	Variant	Variant
Intron	No	Yes	Yes
Promoter	2 major	Multiple	Multiple
tRNA coding	Sufficient	Sufficient	Insufficient
Splicing	No splicing	*Cis- & Trans-*	*Cis- & Trans-*
RNA editing	Very rare	Very rare	Frequent

DNA necessary to account for the coding functions (Fauron et al., 1995a; Unseld et al., 1997). The mitochondrial genomes contain approximately 20-40 protein-coding genes that encode largely for the ATPase subunits for oxidative phosphorylation, three major respiratory enzyme complexes of the electron transport pathway, and the machinery to express mitochondrial genes. Recent publication of the complete nucleotide sequence of the *Arabidopsis thaliana* mitochondrial genome revealed that 60% of the genome is non-coding sequences of unknown origin (Unseld et al., 1997).

A "master circle" has been proposed for the genome structure on the basis of physical mapping from *Brassica campestris* (Palmer and Shields, 1984) and *Zea mays* (Lonsdale et al., 1984). The high frequency of recombination between pairs of repeat sequences on a "master circle" leads to mtDNA rearrangement and duplication contributing to mitochondrial genome expansion in plants (Fauron et al., 1990b; Palmer and Hebron, 1986). More than one autonomous sub-stoichiometric molecule was suggested for the structure of the plant mitochondrial genome in some species (Small et al., 1989; Folkerts and Hanson, 1991; Janska and Mackenzie, 1993).

Recent studies using pulse field gel electrophoresis and moving picture analysis suggested that the actual physical structure of intact mitochondrial

genomes in plants is primarily large linear and branched molecules. Circular mtDNAs smaller than genome size have been observed, but are always rare (Oldenburg and Bendich, 1996; Backert et al., 1996; Bendich and Smith, 1990; Bendich, 1996). Only small amounts of open circles and sigma-like molecules are detected in plant mitochondrial genomes (Bendich, 1993; Backert et al., 1996). Thus, the *in vivo* structure of the large mitochondrial genome and the existence of a master molecule have not been clearly demonstrated.

In a study of cultured tobacco cells, pulse-chase experiments with ^{3}H-thymidine suggested that the newly synthesized mtDNA remains well-bound during electrophoresis. Most of well-bound mtDNA is branched structures of multigenomic concatemers larger than the 270 kb size of the tobacco mitochondrial genome (Oldenburg and Bendich, 1996). Therefore, it was suggested that mtDNA replication in plants could be initiated by recombination.

The plant mitochondrial genome has the ability to undergo homologous recombination between repeats that vary in size from 6 bp up to 14 kb sequences (Lonsdale et al., 1984; Palmer and Shields, 1984; reviewed by Andre et al., 1992). Lonsdale et al. (1984) suggested that the molecular

heterogeneity of the maize mitochondrial genome arises by recombination at repeated regions in the mitochondrial DNA (mtDNA). Due to recombination at repeated sequences, the plant mitochondrial genome could exist in many different configurations varying in stoichiometry (Small et al., 1989). Variation in the relative stoichiometry of different mtDNA configurations has been reported and shown to be widespread in plant cytoplasms (Morgens et al., 1984; Small et al., 1987). Most of the mtDNA exists as molecules generated by recombination through large repeats. However, low abundance (sub-stoichiometric) molecules termed 'sublimons' (Small et al., 1987) are generated by infrequent recombination between small repeats (6-689 bp) according to the recombination model suggested by Small et al. (1989) and reviewed by Andre et al., (1992).

Transcription of Mitochondrial Genes

In plant mitochondria, individual genes can give rise to multiple transcripts. In part, this results from expression of multiple gene copies that vary in flanking sequences. Unlike mammalian mitochondrial genes, plant mitochondrial genes can have multiple promoters giving rise to multiple transcription initiation sites for different sizes of transcripts. Multiple

transcripts resulting from multiple initiation sites (5-6) have been identified for *cox2, cob* and *atp9* genes in maize (Mulligan et al., 1988). Multiple transcripts of rRNAs (26S, 18S, and 5S) result from the endonucleolytic cleavage (Mulligan et al., 1991).

CRTA (R; purine) or YRTA (Y; pyrimidine) consensus sequences have been identified as promoters in higher-plant mitochondria (Mulligan et al., 1991; Covello and Gray, 1991; Brown et al., 1991; Rapp and Stern, 1992; Rapp et al., 1993; Binder et al., 1995; Nakazono et al., 1995). The motifs are conserved in the promoter regions of both monocots [maize (Rapp et al., 1993; Mulligan et al., 1991), wheat (Covello and Gray, 1991), rice (Nakazono et al., 1995)] and dicots [soybean (Brown et al., 1991) and *Oenothera* (Binder et al., 1995)]. These core nucleotide sequences may be essential for the basal transcription activity. However, variations of the YRTA core consensus sequence have been reported in *rrn18* in Arabidopsis (AATA) (Giese et al., 1996), *urf209* (AATA) in Sorghum and *atp6-1* (CTTA) in Sorghum (Yan and Pring, 1997). The complex context of the upstream nucleotide rather than the individual sequence itself appears to be involved in controlling the promoter activity. The functional analysis of the *atpA* promoter in maize and *Oenothera* showed that an 11-bp domain of (A/T)CRTA(G/T)A(A/T)AAA, a

nona-nucleotide region including the basal motif and extending 3', is crucial for transcription initiation (Rapp and Stern, 1992; Binder et al., 1995).

The mRNAs of the plant mitochondria are generally larger than the actual coding regions of the genes because the non-coding 5' and 3' regions of several hundred nucleotides are transcribed with the coding regions. As discussed above, the size of the plant mitochondrial genome greatly exceeds the amount of DNA necessary to account for the essential coding information (Fauron et al., 1995a; Unseld et al., 1997). Thus, most of coding regions in plant mitochondria are separated by up to several kilobases of non-coding spacer sequences. A recent study demonstrated that 3'-terminal inverted-repeat structures regulate mRNA processing and stability in plant mitochondria (Dombrowski et al., 1997). The presence of 3' inverted repeat structures that form stem-loop stabilize upstream sequences of the transcripts.

In some instances, plant mitochondrial genes show co-transcription into discistronic RNA. For instance, the 18S/5S RNA genes in soybean and maize (Morgens et al., 1984; Maloney and Walbot, 1990) or the *nad3-rps12* in wheat and maize (Gualberto et al., 1988) are usually transcribed as a single unit. In the *Phaseolus* mitochondria, *atpA/orf209/pvs* is another example of co-transcription (Chase, 1994).

In higher plant mitochondria, typical *cis*-splicing group II introns have been identified in the protein coding genes *coxII* (Fox and Leaver, 1981), *nad1*(Chapdelaine and Bonen, 1991), *nad4* (Lamattina and Grienenberger, 1991), *nad5*(Knoop et al., 1991),and *rps3* (Hunt and Newton, 1991). In addition, *trans*-splicing reactions occur to give rise to the mature transcripts of the *nad1, nad2*,and *nad5* genes in almost all flowering plants examined (reviewed in Gray et al., 1992). The *trans*-splicing events occur in mitochondria and chloroplasts, but are more common in plant mitochondria. Such *trans*-splicing may be the adaptation to assemble the coding regions dispersed by mtDNA recombination in plant mitochondria.

Cytoplasmic Male Sterility (CMS)

The mitochondria of higher plants are not only the major sites of energy production by oxidative phosphorylation (reviewed by Attardi, 1988), but also have been found to have genes associated with CMS (reviewed by Hanson, 1991). CMS, the failure to produce functional pollen, is inherited through the female parent (Rhoades, 1950). Nuclear fertility restoration genes can suppress or overcome the expression of mitochondrial genes to restore male fertility (reviewed by Hanson and Conde, 1985). CMS systems can be exploited as convenient genetic materials for F1 hybrid seed

production and for molecular investigation of mitochondrial-nuclear interaction in higher plants. In most cases, CMS plants are morphologically normal other than pollen abortion due to flower-specific aberrations (Laser and Lersten, 1972). A rapid and large increase in the number of mitochondria per cell has been observed in the developing microspores and surrounding tapetal tissues of maize perhaps to provide the increased energy demands of microsporogenesis (Warmke and Lee, 1977).

The cytoplasms of maize serve as a model for the CMS and nuclear restoration system in higher plants. The three major types of maize CMS systems (T, S, and C) are distinguished on the basis of specific nuclear restorers (reviewed by Levings, 1993). The *urf13* gene of maize T cytoplasm is the best-characterized of the many chimeric genes that are associated with the CMS phenotype (Dewey et al., 1986). The chimeric *urf13* contains DNA sequences derived from various regions of the mitochondrial genome (Dewey et al., 1985; Dewey et al., 1986). These include the 5' flanking region of the maize *atp6* gene and the coding and 3' flanking sequence of the 26S rRNA gene (*rrn26*). Other chimeric genes that are associated with CMS have been reported in other plant species (reviewed by Hanson, 1991).

Nuclear Fertility Restoration

Nuclear fertility restorer genes restore male fertility by suppressing or counteracting the expression of mitochondrial genes (reviewed by Hanson and Conde, 1985). For instance, nuclear *Rf1* and *Rf2* genes are required to restore fertility in the CMS-T cytoplasm of maize. The *Rf2* gene, which is the only fertility restorer gene to be cloned to date, encodes a putative aldehyde dehydrogenase (ALDH) (Cui et al., 1996). Mitochondrial ALDH, which can oxidize acetaldhyde to acetate, is expressed specifically in the male and female reproductive tissues of tobacco (Camp and Kuhlemeier, 1997). This suggests that ALDH may have an important function for pollen tube growth in tobacco. According to the "metabolic" role of ALDH (Cui et al., 1996), the energy produced by ALDH from the acetaldehyde intermediate of ethanolic fermentation becomes essential if T-cytoplasm tapetum cells have an energy deficit. In addition, ALDH has a potential role in scavenging and detoxifying acetaldehyde produced by ethanolic fermentation flux, which is induced by excessive carbohydrate levels in tobacco pollen (Tadege and Kuhlemeier, 1997).

Other restorer genes reduce the accumulation of mitochondrial CMS gene products in different ways. *Rf1* decreases the accumulation of the T-

urf13 transcripts in maize (Dewey et al., 1987; Kennell and Pring, 1989), whereas the *Fr* gene irreversibly removes the *pvs* mtDNA sequence in common bean (Mackenzie and Bassett, 1987; Mackenzie et al., 1988). Therefore, CMS determinants in mitochondria and restorer genes in the nucleus may not share common metabolic mechanisms in all higher plants.

Mitochondrial RNA (mtRNA) Processing in Fertility Restoration.

Nuclear-encoded fertility restoration genes have been implicated to regulate the expression of mitochondrial CMS genes at the transcriptional and post-transcriptional level. The maize nuclear gene *Rf1,* one of two genes required for fertility restoration of T-cytoplasm (Levings, 1993), is associated with an RNA processing activity internal to T-*urf13* and reduces URF13 protein about 80% (Dewey et al., 1987; Kennell and Pring, 1989). RNA processing activity internal to open reading frames may also be involved in fertility restoration of wheat (Song and Hedgcoth, 1994) and *Brassica pol* (Singh and Brown, 1991) CMS cytoplasms. In the Chinsurah Boro II male-sterile rice cytoplasm, a di-cistronic *atp6-orf79* transcript associated with CMS was processed to mono-cistronic transcripts to restore the fertility in the presence of nuclear restoration gene *Rf1* (Akagi et al., 1994). A chimeric

mitochondrial *orf107* of CMS sorghum consists of sequences similar to *atp9* and to *orf79* of CMS rice. A high-efficiency internal-*orf107* RNA processing activity is correlated with partial or full restoration to fertility in sorghum (Tang et al., 1996). Moreover, the abundance of a 12 kDa *in organello*-synthesized polypeptide in male-sterile sorghum is correlated with differential *orf107* transcript processing activity (Bailey-Serres et al., 1986). This suggests that nuclear-encoded RNA internal processing activity disrupts the gene coding associated with CMS to restore the fertility.

Nuclear restorer alleles can specifically affect the transcripts of mitochondrial genes during pollen development, and male-sterility can be restored to fertility by the differential regulation of mitochondrial transcription. In sunflower CMS systems, steady-state RNA levels of mitochondrial genes, *atpA, atp9, cob, and rrn26*, increase in meiocyte and tapetal cells (Smart et al., 1994). In CMS-S maize systems, presence of a nuclear restorer for fertility (*Rf3*) not only decreased abundance of CMS-unique transcripts in developing microspore, but also created the novel shorter transcripts during pollen development (Zabala et al., 1997; Wen and Chase, submitted). It does, however, remain to be seen exactly how fertility

restoration genes regulate the mitochondrial gene expression and restore fertility from CMS lines.

CMS in *P. vulgaris*

In *P. vulgaris*, mitochondrial DNA (mtDNA) sequences (3,736 bp) designated *pvs* (for ***Phaseolus vulgaris*** sterility) are associated with CMS (Chase and Ortega, 1992; Johns et al., 1992). The *pvs* region is flanked by two different repeat sequences x and y in a configuration we designate the *config-pvs*. Configurations designated *config-rx* and *config-ry* contain other copies of repeat x or repeat y, respectively, (Fig. 1-1). The repeat x includes a complete copy of the F1 ATPase subunit A (*atpA*) gene and an overlapping open reading frame (*orf209*). An apocytochrome b (*cob*) gene segment (117 nt) is located in repeat y (Chase and Ortega, 1992).

At least two different nuclear fertility restoration systems have been identified for the *P. vulgaris* cytoplasm (Mackenzie and Bassett, 1987; Mackenzie, 1991). The nuclear fertility restorer gene designated *Fr* irreversibly alters the mitochondrial genome structure by eliminating the *pvs* sequence (Mackenzie and Bassett, 1987; Mackenzie et al., 1988). In contrast, another fertility restorer *Fr2* does not alter the mitochondrial genome so that

the *pvs* sequence is present in the *Fr2* restored line (G08063) as well as the CMS line (CMS-Sprite) (Mackenzie, 1991). The *config-pvs* is also lost upon spontaneous cytoplasmic reversion to fertility, the mitochondria of the WPR-1 cytoplasmic revertant line do not carry *config-pvs* (Mackenzie and Chase, 1990; Mackenzie et al., 1988), but still retain *config-ry* and *config-rx* (Chase and Ortega, 1992).

The *pvs* and related mitochondrial transcripts have been characterized by northern analysis (Chase, 1994). Northern hybridization revealed CMS-unique *pvs* transcripts of 7.0, 6.8, 4.7, 3.3, and 2.8 kb. The 7.0 kb is the most abundant transcript, and sedimentation assays indicate that the 7.0 kb transcript is associated with polyribosomes (Chase, 1994). A 2.1 kb segment of the 7.0 kb transcript is not accounted for by northern hybridizations with the *pvs* and flanking DNA clones (Chase, 1994). It was suggested that RNA splicing processes and/or novel DNA configurations (perhaps a sublimon) could explain for the origin of the unaccounted 2.1 kb in the CMS-unique 7.0 kb transcript (Chase, 1994). In the *pvs* region, there are two open reading frames, *orf98* and *orf239.* The *orf239,* of unknown origin, is the longest open reading frame (Chase and Ortega, 1992; Johns et al., 1992). The *orf98* and

orf239 are co-transcribed with the upstream *atpA* gene and an overlapping *orf209* to give rise to the 6.8 kb *pvs* transcript (Chase, 1994).

Immuno-histochemical examination demonstrated that the ORF239 protein is expressed only in male reproductive tissues of CMS lines during anther development (Abad et al., 1995). Transgenic tobacco expressing *orf239* gene constructs are male sterile and have cell wall depositions of the ORF239 protein, suggesting that ORF239 is responsible for CMS and may act outside of the mitochondria (He et al., 1996).

RNA Editing

RNA editing refers to the post-transcriptional alteration of the RNA nucleotide sequences from those encoded by the DNA template (reviewed by Gray and Covello, 1993; Pring et al., 1993). It was first discovered as an uridine deletion/insertion system in trypanosome mitochondria (Benne et al., 1986). In higher plant mitochondria, C residues of genomic clones have been replaced by T residues in cDNA clones (Covello and Gray, 1989; Gualberto et al., 1989; Hiesel et al., 1989). C to U conversion occurs predominantly in the mitochondria of higher plant (Covello and Gray, 1989; Gualberto et al., 1989; Hiesel et al., 1989), and less frequently in chloroplasts (Hoch et al.,

1991) and in the nuclear-encoded, mammalian apolipoprotein-B RNA (Hodges and Scott, 1992). In plant mitochondria, most protein-encoding RNAs examined are edited by C to U conversion and occasionally by U to C conversion (Gualberto et al., 1990). RNA editing occurs mainly in the protein coding region of mRNA; however 5' and 3' untranslated region and introns have been found to be edited (reviewed by Gray and Covello, 1993; Pring et al., 1993). Most RNA editing events change amino acids sequences to enhance the amino acid conservation of the encoded proteins (reviewed by Gray and Covello, 1993; Pring et al., 1993). However, silent editing is also observed (Kempken et al., 1995). In wheat, an initiation codon is created by editing the *nad1* transcript (Chapdelaine and Bonen, 1991), and termination codons are created by editing the *atp9* and *rps1* transcripts (Begu et al., 1990; Gonzalez et al., 1993). Using the chloroplast *in vitro* translation systems, Hirose and Sugiura (1997) showed that translation initiates only from the AUG initiation codon of *ndhD* (NADH dehydrogenase subunit 4) that is created by RNA editing in tobacco chloroplast.

Although the specificity mechanism of RNA editing remains unknown, studies show that the sugar-phosphate backbone of the RNA is retained without any breakage during the editing process, suggesting that the ribosyl

residues are deaminated or transglycosylated for the conversion (Rajasekhar and Mulligan, 1993). Recent evidence for a site-specific deamination of cytidine was revealed in C to U RNA editing of plant mitochondria (Yu and Schuster, 1995). RNA editing of chimeric *atp6* transcripts from CMS-C maize is similar to that of normal *atp6* transcripts from sorghum, suggesting that RNA editing is not influenced by mtDNA rearrangement but instead is sequence specific (Kumar and Levings III, 1993). Schuster and Brennicke, (1991) explained partial editing events on silent sites as a random switching that should be tolerated.

The extent of RNA editing varies among plant mitochondrial transcripts. The *atp9*, *coxII,* and *coxIII* transcripts are completely edited in all species examined, whereas petunia *atp6* (Lu and Hanson, 1994), potato *nad9* (Grohmann et al., 1994), *nad3* (Grosskopf and Mulligan, 1996) and *rps12* (Phreaner et al., 1996) transcripts are usually incompletely edited. Incompletely editied *rps12* transcripts are translated in maize mitochondria, resulting in polypeptide polymorphism (Phreaner et al., 1996). The mRNA heterogenity as a result of incomplete editing may be related to relative promoter strength and rate of editing (Mulligan et al., 1991). Recent study of *nad3* and *rps12* cDNA suggested that the overall extent of editing is affected

by the nuclear genotype but not by the transcriptional context. Wilson and Hanson (1996) indicate that non-silent editing sites (which change amino-acids) are more frequently edited than silent sites in all the organisms examined.

The degree of RNA editing can change with developmental or growth conditions (Grosskopf and Mulligan, 1996). Analysis of three editing sites in the *nad3* cDNA of maize species indicated that the degree of partial editing increased from 50% at 3 days to about 75% at 7 days in the mitochondria of maize seedlings, and was 85% in suspension-cultured cells (Grosskopf and Mulligan, 1996). In the tobacco chloroplast *ndhD* transcript, the level of edited transcripts is higher (56%) in photosynthetically active tissues like green leaf. In the dark-grown seedling, the extent of edited *ndhD* transcripts was reduced to 34% (Hirose and Sugiura, 1997). Thus, the extent of C to U editing varies on a temporal and environmental basis.

Many mitochondrial genes associated with CMS have been studied for RNA editing. The unedited *atp9* sequence of wheat mitochondria induced male-sterility in transgenic tobacco plants when a nuclear-encoded, unedited ATP9 polypeptide was imported into mitochondria (Hernould et al., 1993). In the rice *atp6* mitochondrial gene, frequency of RNA editing was higher in

a fertility restored line than in a CMS line, suggesting differential RNA editing between two lines (Iwabuchi et al., 1993). In *Sorghum bicolor*, mitochondrial *atp6* RNA editing was strongly reduced in anthers of the male-sterile line, compared to etiolated seedlings of the same line. Moreover, restoration of fertility correlated with an increase in RNA editing of *atp6* transcripts in the anther (Howad and Kempken, 1997). This suggests that tissue-specific loss of *atp6* RNA editing is associated with CMS in *Sorghum bicolor*. On the other hand, transcripts of the chimeric *orf522* gene in sunflower mitochondria are associated with CMS and completely edited in both CMS and restored lines (Moneger et al., 1994). Transcripts of the maize T-*urf13* gene (Ward and Levings III, 1991) and the *urfS* portion of the petunia *pcf* gene (Nivison et al., 1994), which are associated with CMS, are not edited. The *orf221* co-transcribed with T-*urf13* is edited at 12 sites, but the *orf221* is not associated with CMS (Ward and Levings III, 1991). Therefore, there is no evidence suggesting that reduction of RNA editing frequency in plant mitochondria is always related to the CMS phenotype.

Materials and Methods

Plant Material

The lines of common bean (*P. vulgaris*) used in this study are presented in Table 1-2. CMS-Sprite is a CMS line with the *pvs* sequence in a non-restoring nuclear background (maintainer). CMS-Sprite was derived from accession line G08063 by back-crossing to the 'Sprite,' snap bean cultivar (the source of nuclear maintainer). G08063 is a fertility-restored line, carrying the nuclear restoring allele *Fr2* in the presence of the sterility inducing cytoplasm. WPR-1 is a heritable, cytoplasmic revertant spontaneously derived from CMS-Sprite. CMS Mo-Triumph was derived from *P. coccineus* Morelos 662 cytoplasm with a sterility-maintainer of nuclear background (*P. vulgaris* MM3).

Table 1-2. Genetic Materials

Line	Phenotype	MtDNA Configurations	Nucleus	Source
CMS-Sprite	male-sterile	*rx, ry and pvs*	non-restoring *fr2/fr2*	Bassett & Shuh, 1982.
WPR-1	male-fertile	*rx and ry*	non-restoring *fr2/fr2*	Mackenzie et al., 1988.
G08063	male-fertile	*rx, ry and pvs*	restoring *Fr2/Fr2*	Mackenzie, 1991.
Mo-triumph	male-sterile	*pvs and ry*	non-restoring *fr2/fr2*	Hervieu et al., 1993

Isolation of Mitochondria and Extraction of Nucleic Acids

Mitochondria were purified from 5-7 day old etiolated cotyledons and hypocotyls by differential centrifugation and lysed in the presence of SDS. MtDNAs were purified from the isolated mitochondria as described by Wilson et al. (1989). For the preparation of mtRNA, mitochondria were lysed in 6M guanidine thiocyanate; RNA was purified by extraction with phenol and chloroform, followed by precipitation with isoamylalcohol as described by Chase (1994).

Gel Electrophoresis, Nucleic Acid Transfer, and Blot Hybridization

DNA samples were digested with restriction endonucleases, fractionated by agrose gel electrophoresis and transferred to nylon supports (Hybond-N, Amersham Corp.) as described by Wilson et al. (1989). The fractionated DNA was transferred to nylon membranes by capillary blotting (Sambrook et al., 1989) and cross-linked by baking (1h at 80°C). The mtRNAs were denatured in glyoxal, separated by agarose gel electrophoresis, and transferred to a nylon membrane as described by Chase (1994). Southern and Northern blot hybridization experiments were carried out by the methods described in Chase, (1994) except for modified buffers

and hybridization conditions. SSPE buffer (5X; 0.9 M NaCl, 50 mM NaH_2PO4, and 5mM Na_2EDTA) in Denhart's solution (5X; 0.1% w/v BSA, 0.1% w/v Ficoll400, and 0.1% w/v PVP360) with 0.5% w/v SDS and 100 mg/ml sheared salmon sperm DNA were used for hybridization. SSPE buffer (0.1X) with 0.1% SDS were used for washing as described in the manufacture's protocol (Amersham Corp.). Hybridizing and washing temperatures were maintained at $60^{o}C$. The DNA template for labeling was purified from either PCR products or inserts recovered from cloned plasmid DNA. Templates were purified from agarose as described by Chase (1994). Purified DNA fragments were labeled by RTS RadPrime DNA labeling system (Life Technologies) in the presence of [α-^{32}P]dCTP (Feinberg and Vogelstein, 1983).

cDNA Synthesis and Amplification

First strand cDNAs were reverse-transcribed from the mtRNAs with Superscript reverse transcriptase (Life Technologies) and sequence-specific oligonucleotides. The first-strand cDNAs synthesized were subsequently amplified by polymerase chain reaction (PCR) with *Taq* DNA polymerase (Life Technologies). The thermocycler program for PCR amplification was a

pre-denaturation at 92°C for 2 min followed by 35 cycles of 92°C (35 s) for denaturation, 60°C (45 s) for annealing, and 72°C (1.5 min) for synthesis. The PCR products were purified from low-melting agarose (GTG) gels and then ligated into the pGEM-T plasmid vector (Promega) by utilizing the A-overhang of PCR products. The ligated DNAs were transformed into DH5α competent cells purchased from Life Technologies. PCR amplification products from genomic mtDNA were also sequenced to verify the previously published sequences and editing sites found in the cDNA examined.

List of Primers

Oligonucleotides specific to gene of interest were synthesized by the DNA Synthesis Core of the Interdisciplinary Center for Biotechnology Research at the University of Florida or by Gibco BRL Custom Primers from Life Technologies. Position numbering of primers starts from the first base of the predicted translation start codon in the *atpA* gene (Chase and Ortega, 1992). The primers used for the synthesis of cDNA are listed in Table 1-3.

Sequencing of DNA and cDNA

All the nucleotide sequences were obtained from double-stranded plasmid DNA and sequenced on both strands by the dideoxy nucleotide

chain-termination method (Sanger et al., 1977) with Sequenase version 2.0 (U.S. Biochemical Corp.). Isolated plasmid DNA templates were denatured in 2 M NaOH before annealing primers. Synthetic oligonucleotide primers were annealed to the denatured template DNAs and extended by DNA polymerase, Sequenase version 2.0 in the presence of [α-^{32}P]dCTP. The labeled extension products were fractionated by electrophoresis at 55 watts for 2 to 5 hours on 8% polyacrylamide-bisacrylamide/8M urea gels in 1X TBE buffer and autoradiographed on Kodak diagnostic film X-Omat™ XRP-5.

Results

Northern Hybridization Detected CMS-Unique *atpA/orf209* RNAs.

Modified restriction maps surrounding the *pvs* region were generated (Fig. 1-1) on the basis of the previous mtDNA maps (Chase, 1994). The CMS-inducing mitochondria in CMS-Sprite and G08063 carry the *config-pvs* as well as *config-rx* and *config-ry*. Mitochondria of the WPR-1 revertant line do not carry *config-pvs*, but still retain *config-ry* and *config-rx*; the *config-pvs* is lost upon reversion to fertility (Mackenzie et al., 1988; Chase and Ortega,

1992). In contrast, it has been found that CMS-inducing mitochondria in the Mo-Triumph line only carry *config-pvs* and *config-ry* (Hervieu et al., 1993).

Three abundant transcripts (3.0, 2.8 and 2.5 kb) were shown to be transcribed from *atpA/orf209* coding sequences (Mackenzie and Chase, 1990). The 3' termini of these transcripts were localized downstream of the *orf209* termination codon (Chase, 1994). As the downstream sequences of *atpA/orf209* mtDNA in *config-pvs* are different from those in *config-rx* for the *atpA/orf209* transcripts, the 3' regions of the *atpA/orf209* transcripts may be different for transcripts derived from *config-pvs* and from *config-rx*. This also suggested that CMS-unique *atpA/orf209* transcripts might be identified with *pvs* region probes.

Northern experiments were performed by sequential hybridizations with DNA probes (A, B, and C in Fig. 1-1) spanning *atpA* and *pvs* to RNA blots of CMS-Sprite, Mo-Triumph, and revertant WPR-1. The probes B (308 bp) and C (450 bp) were derived from the *pvs* sequences, which were 3' flanking to the *atpA/orf209* coding sequence in *config-pvs*. Thus, the probes B and C are only present in CMS lines (CMS-Sprite and Mo-Triumph) whereas the probe A in the *atpA* coding region is present in both CMS and revertant lines. Particularly the probe B was designed to identify 3' termini

of *atpA/orf209* transcripts in *config-pvs*. Hybridization revealed CMS-unique versions of *atpA/orf209* transcripts in CMS lines (Fig. 1-2) in addition to the previously reported *atpA/orf209* transcripts observed in all lines (Chase, 1994). Signal intensities are not necessarily comparable between lanes of Fig. 1-2. The probe B hybridized the abundant transcripts of *atpA/orf209* in CMS lines (3.0, 2.8, and 2.5 kb in CMS-Sprite; 3.0 and 2.5 kb in Mo-Triumph), but failed to hybridize any transcript in WPR-1. This result confirmed *atpA/orf209* transcripts unique to the CMS lines and placed the 3' termini of these transcripts in the *pvs* region of *config-pvs*.

Two CMS-unique *atpA* transcripts (3.0 and 2.5 kb) were identified by probe B in Mo-Triumph, and one 2.8 kb CMS-unique transcript was not detected in this line. This could be because of a different nuclear background, or altered DNA sequences in the Mo cytoplasm. Further investigation will be required to determine the basis for this observation.

Hybridization of probe C confirmed the presence of the CMS-unique *pvs* transcripts (7.0, 6.8, 4.7, 3.3, and 2.8 kb) in CMS lines (CMS-Sprite and Mo-Triumph) as shown in previous studies (Chase, 1994; Hervieu et al., 1994), and hence served as a control for the Northern analysis (Fig. 1-2). Hybridization of the probe A also confirmed three abundant *atpA/orf209*

transcripts (3.0, 2.8, and 2.5 kb) found in the WPR-1 by Chase, (1994), indicating that these transcripts were derived from the *atpA/orf209* sequences which had 3' termini in downstream sequences of *config-rx*.

RNA Editing of the *atpA/orf209* Transcript

Several cDNA clones (designated pGEM in Fig. 1-1) covering the *pvs* and flanking sequences were generated by RT-PCR from mtRNA to study RNA editing. Amplification from the residual mtDNA was rule out by negative controls lacking reverse transcriptase. The CMS-unique mtDNA configurations in *P. vulgaris* offered advantages for the study of RNA editing. As shown Fig. 1-1, two different repeats (x and y) flanked the *pvs* sequences. Therefore, comparison of cDNA synthesized from *config-pvs* and *config-rx* or *config-ry* would address potential RNA editing specificity and determinants. In addition, examinations of cDNA generated from the CMS-unique transcripts might give insight into any role of RNA editing in this CMS system.

To determine whether RNA editing could generate a novel orf or create new start or stop codons in the CMS-unique *atpA* transcripts, cDNA clones covering *atpA/orf209* locus were examined. Sequencing of cDNA clones

(pGEM8173) derived from CMS-unique *atpA/orf209* transcripts in *config-pvs* revealed three C-to-U RNA editing events within the *atpA* open reading frame, and one C-to-U editing within *orf209.* RNA editing in the *atpA* included one partial (position 1029) and two complete (position 1166 and 1523) edits (Fig. 1-3). The partial editing C^{1029}-to-U did not alter the deduced amino-acid sequence, while two complete edits, C^{1166}-to-U and C^{1523}-to-U, resulted in amino-acids changes Ala to Val and Thr to Ile, respectively (Fig. 1-4). In *orf209*, one partial editing event (C^{797}-to-U) was observed from one cDNA clone (pGEM8173-2) out of seven, and predicted alteration of a deduced amino-acid, Pro to Ser (Fig. 1-4). It is not clear if this represents low-level editing activity or *Taq* polymerase error. Since there is a possibility that single nucleotide alteration might have been experimental artifact, we need to verify that site with other independent cDNA clones. RNA editing events were not observed for the 3' flanking sequence of *atpA/orf209* transcripts in the *config-pvs* or *config-rx*, eliminating the possibility that RNA editing could create a new novel ORF or a new start or stop codon in this region.

RNA editing patterns of the CMS-unique *atpA/orf209* transcripts were compared with those of *atpA/orf209* transcripts from *config-rx* by sequencing

cDNA (pGEM3766) (Fig. 1-3). Similar editing patterns were observed in *config-rx* and *config-pvs* transcripts. No editing sites were observed in *orf209* cDNA of *config-rx* (pGEM3766), while one partial editing site was found in *orf209* cDNA of *config-pvs* (pGEM8173). If the one partial editing event found in pGEM8173 is a true edited site, RNA editing may be changed in different context, and primary sequence, 3' flanking region in this case, can contain determinant(s) for editing.

RNA Editing of *orf239* and the *cob* Segment in *config-pvs*

Sequencing of cDNA clones (pGEM8155) derived from CMS-unique *pvs* transcripts in *config-pvs* revealed three C-to-U RNA editing events within the *orf239* coding region. The cDNA (pGEM8155) amplified by sequence-specific oligonucleotides (*cc78* and *cc44*) covered the entire *orf239* and 3' *cob* segment in the *config-pvs*.

The edited sites and the corresponding genomic sequences are shown by autoradiographs of sequencing gels (Fig. 1-5) and represented by diagram in Fig. 1-6. RNA editing in the *orf239* included three partial edits (position 5267, 5291, and 5811). None of these edits changed the encoded amino-acids. The deduced amino-acids are Ser at C^{5267}-to-U, Phe at C^{5291}-to-U, and

Leu at C^{3811}-to-U (Fig. 1-7). The frequency of each edited site examined in ten cDNA clones was 60% for C^{3267}-to-U, 40% for C^{3291}-to-U, 50% for C^{3811}-to-U (Fig. 1-6).

RNA editing of the *orf239* was examined in CMS-Sprite and the *Fr2* restored line (G08063) for potential variation, as the fertile G08063 retained the *pvs* sequence and transcripts. A total five cDNA clones from CMS-Sprite and five from restored G08063 RNAs were analyzed. No notable difference of RNA editing was revealed between CMS-Sprite and G08063 in terms of frequency and pattern (Fig. 1-6).

In the cDNA (pGEM8155), the *cob* gene 3' segment co-transcribed with the *orf239* of *config-pvs* contained three sites were completely edited. All of these edits resulted in changes of amino-acid sequence. The deduced amino-acid changes were Pro to Ser at C^{3936}-to-U and C^{3923}-to-U, and Pro to Leu at C^{3966}-to-U (Fig. 1-7). Since the *cob* segment in the *config-pvs* is repeated in the complete *cob* gene copy of the *config-ry*, we were able to compare the editing patterns of these two repeated regions. The 3' end of the *cob* gene was sequenced from pGEM8156 cDNA clones and compared to the 3' *cob* segment in pGEM 8155 clones. Sequencing of pGEM8156 cDNA clones derived from the coding copy of the *cob* gene in *config-ry* revealed that the

editing patterns within the *cob* gene segment of config-*pvs* were the same as those within the config-*ry* coding copy of the *cob* gene in (Fig. 1-6). This demonstrated that the 117 nt *cob* segment, out of context, contained all information required for editing. No editing event was found in single representative cDNA clones (pGEM7772 and pGEM8049) of the remaining *pvs* region, precluding high-frequency editing in non-coding *pvs* sequences.

Homologs of *orf239* Were Present in Other Plant Species.

Three RNA editing events found in *orf239* raised the possibility that *orf239* could be part of an as-yet-unidentified mitochondrial protein coding gene in plants. Recent evidence for tissue-specific expression of ORF239 protein supports this possibility (Abad et al., 1995). Southern analysis showed that *orf239*-homologous sequences were found in total genomic DNAs of soybean, broad bean, crimson clover, tobacco, watermelon, and peanut (Fig. 1-8). The *orf239* homologous sequence was transcribed in soybean and broad bean mitochondria (Fig. 1-9). However, no *orf239*-homologous sequences were identified in maize, lupinus, or Arabidopsis.

Discussion

CMS-Unique *atpA* Transcripts

In this study, I have characterized the *atpA/orf209* locus from CMS and revertant cytoplasms in *Phaseolus* species in order to gain further information on sequences involved in the CMS phenotype. Previous study revealed three abundant *atpA/orf209* transcripts (3.0, 2.8, and 2.5 kb) in CMS and revertant lines (Mackenzie and Chase, 1990). Consistent with this observation, *atpA/orf209* transcripts (3.0, 2.8, and 2.5 kb) were verified in CMS-Sprite and WPR-1. However, additional *atpA/orf209* transcripts (3.0, 2.8, and 2.5 kb) were found to be unique in CMS-Sprite. The sizes and patterns of these transcripts were similar to those detected previously. The *atpA/orf209* transcripts unique to CMS were derived from *config-pvs*, and 3' termini of these transcripts were placed in the probe B segment (308 bp) of the *pvs* region. In contrast, the 3' termini of the *atpA/orf209* transcripts in WPR-1 were derived from *config-rx* and were placed upstream and downstream of the junction region in *config-rx* (Chase, 1994).

Since the 3' termini of CMS-unique *atpA* transcripts from *config-pvs* were different from those of *atpA* transcripts from *config-rx*, characterization

of 3' termini for all *atpA* transcripts (at the nucleotide sequence level) may be important to understand transcription termination in plant mitochondria. The finding of *atpA/orf209* transcripts with CMS-unique downstream sequence might suggest that these transcripts are potential candidates for mitochondrial dysfunction at the reproductive stage, resulting in the CMS phenotype. There is a report that unique sequences located downstream from rice mitochondrial *atp6* may cause male sterility in rice (Akagi et al., 1994). However, previous studies have shown that ORF239 protein is only expressed in reproductive tissues (Abad et al., 1995). In addition, the ORF239 protein induces male-sterility in transgenic tobacco (He et al., 1996), demonstrating that ORF239 is responsible for CMS. Thus, the biological role, if any, of these CMS-unique *atpA/orf209* is not known.

Two CMS-unique *atpA* transcripts (3.0 and 2.5 kb) were found in Mo-Triumph by probe B, and one (2.8 kb) transcript was not detected. This could be because of different nuclear background since Mo-Triumph and CMS-Sprite are not iso-nuclear. Therefore, it suggested either that the nucleus could affect the transcription of mtDNA or that initiator or terminator sequences of *atpA* may be altered in Mo-Triumph. As CMS-Sprite and Mo-Triumph are not iso-genic lines, further investigation remains to determine

how nuclear gene(s) interact with mitochondria to influence transcription of mtDNA, or whether the promoter or termination sequences of *atpA* are altered in the Mo cytoplasm.

RNA Editing Improves Protein Conservation.

cDNA clones covering *atpA* and *cob* segments were examined for RNA editing. RNA editing in the 3' coding region of *atpA* included one partial (position 1029) and two complete (position 1166 and 1523) edits. The partial editing C^{1029}-to-U did not alter a deduced amino-acid sequence, while two complete edits, C^{1166}-to-U and C^{1523}-to-U, resulted in amino-acids changes Ala to Val and The to Iso, respectively (Fig. 1-4). Three editing sites of *atpA* in common bean were compared with those sites in sugar beet and three other editing sites of the *atpA* in sugar beet were compared with those sites in common bean (Table 1-4). These genes did not share any common editing sites. The three editing sites in common bean are pre-edited in sugar beet mtDNA, and the three editing sites in sugar beet are pre-edited in common bean.

In the cDNA of the 3' *cob* segment from the *config-pvs*, three sites were almost completely edited. All of these edits resulted in changes of

amino-acid sequence. The editing sites of the *cob* segment were shared with those of other plant species (Table 3). In potato and *Oenothera*, sites 5920 and 5966 are pre-edited in genomic DNA. In wheat, site 5923 is pre-edited. The reason that edited sites differ from species to species may be differences among the primary nucleotide sequences. Amino acids changes resulting from RNA edits of the *P. vulgaris atpA* and the *cob* genes improve the similarity of deduced polypeptide sequences in plants, as has been described in other examples (reviewed by Maier et al., 1996; Hanson et al., 1996).

Silent Editing Is Partial.

We found partial editing patterns at silent editing sites. Silent editing in *orf239* included three partial edits (position 5267, 5291, and 5811), and in *atpA* included one partial edit (position 1029). This was consistent with the previous observations that silent editing of genes in plant mitochondria is partial with various frequencies (Table 1-6). During the evolutionary process, protein conservation is maintained by RNA editing and the silent editing pattern of partial edits would not necessarily be required for protein function. Evolutionary constraints on RNA editing of silent sites were first suggested by Schuster and Brennicke, (1991). Kempken et al., (1991) suggested that

transcripts partially edited at silent sites might be considered as secondary editing events not subject to the constraints of primary, active, editing. Therefore, silent RNA editing might be the result of a rather low error rate of RNA editing in higher plants (Kempken et al., 1995). There is another possibility that a certain mechanism might recognize protein sequence and repair gene expression at RNA level by proofreading mechanism. Further study is required to determine the repair mechanism of plant mitochondria.

RNA Editing is Sequence-Specific.

We have examined cDNAs for RNA editing of the *pvs* and the flanking regions in mitochondria of *P. vulgaris*. Three partial RNA edits of the *orf239* and three complete edits of the 3' *cob* segment (117 nt) are all derived from the same *pvs* transcripts. This indicated that the partial and complete editing patterns can occur on the same transcript. Therefore transcript abundance or stability may not affect the frequency of RNA editing. The frequency of RNA editing would rather be primary sequence-specific. A recent study demonstrated that transcript abundance was independent of RNA editing in *Sorghum bicolor* (Kempken and Howad, 1996). In contrast, there are some studies demonstrating the correlation of transcription rate and transcript

turnover with RNA editing. A single nuclear gene simultaneously affects RNA editing frequency and transcript abundance in petunia (Lu and Hanson, 1992). Promoter strength of mitochondrial genes in maize was related to the extent of RNA editing (Mulligan et al., 1991). More abundant transcripts were more highly edited. However, the overall extent of editing is affected by nuclear genotype, but not by the transcriptional context (Lu and Hanson, 1992; Wilson and Hanson, 1996).

Since the *cob* segment in the *config-pvs* is repeated in the complete *cob* gene copy of the *config-ry*, we were able to compare the editing patterns of these two repeated regions. Sequencing of pGEM8156 cDNA clones derived from coding copy of the *cob* gene in *config-ry* revealed that the editing patterns of the *cob* segment in the *pvs* were the same for three complete editing sites as those of coding copy of the *cob* gene in *config-ry*. It suggested that the 117 nt *cob* segment and 3' downstream sequence out of context contain all information required for editing and thus RNA editing of the *cob* segment is sequence specific. This result was consistent with other examples of sequence-specific RNA editing (Kumar and Levings III, 1993; Chaudhuri et al., 1995).

We had another chance to determine if RNA editing was site-specific. RNA editing patterns of the CMS-unique *atpA/orf209* transcripts derived from *config-pvs* were compared with those of *atpA/orf209* transcripts from *config-rx* by sequencing cDNA (pGEM3766) (Fig. 4). No editing site was observed in *orf209* cDNA of *config-rx* (pGEM3766), while one partial editing site was found in one *orf209* cDNA clone out of seven derived from the *config-pvs* (pGEM8173). If one partial editing event found in pGEM8173 is a true edited site, it will raise the possibility that RNA editing can be changed in different context, and primary sequence, 3' flanking region in this case, can contain determinant(s) for editing.

From this analysis of RNA editing events in CMS-unique transcripts of *P. vulgaris,* we conclude that the determinants of RNA editing reside in the primary sequence. The analysis of numerous additional cDNA clones would be required to determine whether RNA editing can be altered by the specific 3' flanking sequences.

The Origin of *orf239*

Three RNA editing events found in *orf239* raise the possibility that the *orf239* could be part of an as-yet-unidentified mitochondrial protein coding

gene in plants. Southern analysis showed that the *orf239*-homologous sequences were found in total genomic DNA of soybean, broad bean, crimson clover, tobacco, watermelon, and peanut (Fig 1-8). The abundance of these DNA configurations was consistent with an organelle location for these sequences. The finding of the *orf239*-homologous sequences in the tobacco mitochondria (data not shown) is in contrast to the finding of *orf239*-homologous sequences in the *N. tabacum* nuclear genome (Abad et al., 1995). However, it could be possible that the *orf239*-homologous could be in both places, nucleus and mitochondria. Further examination is required concerning location of the *orf239* homologous sequence in tobacco.

The *orf239*-homologous sequence was transcribed in soybean and broad-bean mitochondria as shown in Fig. 1-9. The *orf239*-homologous sequence may also be transcribed in other species. However, the *orf239*-homologous sequence was not identified in maize, lupinus, and Arabidopsis. The *orf239* could potentially have been transferred to nucleus from mitochondria in these species. C*ox2* (Nugent and Palmer, 1991), ribosomal protein *rps12* (Grohmann et al., 1992), and *rps10* (Wischmann and Schuster, 1995) are all found in the mitochondria of some species and nuclear genome of other plant species. As several chloroplast derived tRNA genes have been

identified in plant mitochondria (Joyce and Gray, 1989), we can not rule out the possibility that the *orf239* could be derived from chloroplast sequences. Further characterization of the *orf239* sequences in other species will be required to resolve the questions of its origin and functions.

Table 1-3. Primers used for synthesis of cDNA/DNA fragments.

Synthesized DNA*	Primer	Sequence	Position**	Orientation***
pGEM8048	cc39	5'-GAAGGGCTAGACAGA	-750	Sense
(1130 bp)	cdc39	5'-CGACACGTCTTCGCT	380	Antisense
pGEM8049	cdc32	5'-GCGGGAGACGTATCG	1015	Sense
(1234 bp)	cc68	5'-CCACTGCCTACATAACTAGACTCCC	2249	Antisense
pGEM8155	cc44	5'-GGAGAGCAGGTACAG	4658	Sense
(1368 bp)	cc78	5'-CGTAAATGATTGGTGTC	6026	Antisense
pGEM8156	cc72	5'-TCACATCTAAGTCTCATCCGTAG	5998	Sense
(~ 850 bp)	cc69	5'-CAAGCTTAGCTAGCGCCATACCTG	*cob* gene	Antisense
pGEM8173	cdc45	5'-GATCAAGGGTACTGC	-105	Sense
(1470 bp)	cdc11	5'-GGCATTCGATCACAG	1365	Antisense
pGEM7771	cc10	5'-GCGCTGTGACAAAGAC	2552	Sense
(1167 bp)	cc48	5'-GGGTGGCTCACTTTT	3719	Antisense
pGEM7772	cc47	5'-CGGAGATAAGGTCAG	3321	Sense
(1996 bp)	cc34	5'-CTCAAATCAATGCCG	5317	Antisense
pGEM3766	cdc34	5'-AGTCGCCGCCTTTGC	1205	Sense
(850 bp)	cdc46	5'-GTTAGAACTGCTTGG	*config-rx*	Antisense
Probe A	cc9	5'-GATGGGATTGTACGCG	112	Sense
(1546 bp)	cdc6	5'-GAATCCACCAAGCAA	1658	Antisense
Probe B	cc42	5'-CAGAATACTTCAGGG	2169	Sense
(308 bp)	cc54	5'-CCATCAATAAACCTTATACGCTTG	2477	Antisense
Probe C	cc10	5'-GCGCTGTGACAAAGAC	2552	Sense
(450 bp)	cc11	5'-TGCCTAAAGGACCCC	2902	Antisense
Sequencing	Neb 1211	5'-CGGAAGATTGAGTTCCTG	T3 primer	
Sequencing	cc33	5'-CAGGAAACAGCTATGACC	T7 primer	

*: See Fig. 1-1

**: 5' base of the oligonucleotide; numbering from Chase & Ortega, 1992

***: Sense and antisense relative to *atpA* gene coding

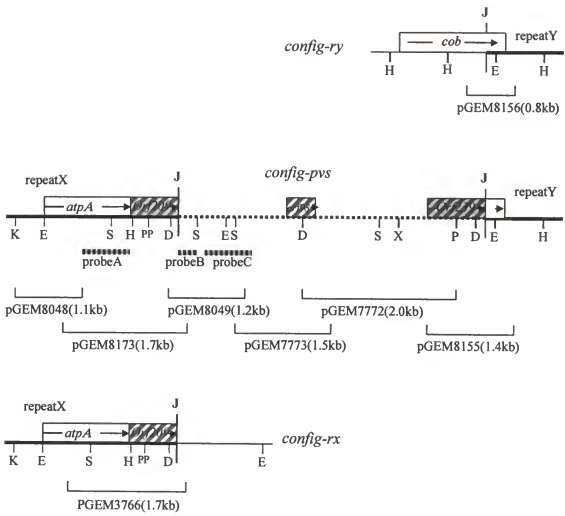

Fig. 1-1. Mitochondrial DNA configurations of the CMS-unique *pvs* region and flanking repeats. The CMS-unique *pvs* sequence (*dashed line)* is flanked by two repeats (*solid lines*) in the *config-pvs*. Junctions designated J represent divergence points between the repeat and the *pvs* sequence in each configuration. Restriction recognition sites are designated as: *Dra*I (D), *Eco*RI (E), *Hind*III (H), *Kpn*I (K), *Pst*I (P), *Xho*I (X), and *Sst*I (S). The diagonally striped boxes represent the *orfs* of unknown origin. The open boxes represent known genes. *Arrows* indicate the 5' to 3' orientations of *orfs* and genes. Dashed boxes placed below the restriction maps indicate DNA probes A, B, and C. The *horizontal lines* labeled with pGEM indicate cDNAs cloned into pGEM-T vectors.

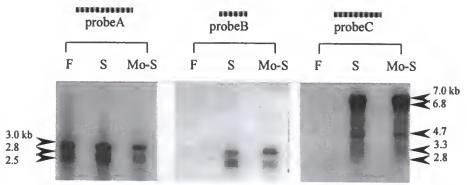

Fig. 1-2. RNA gel blot analysis of the *pvs* and *atpA/orf209* transcripts in CMS lines and male-fertile revertant lines. CMS lines, CMS-Sprite and Mo-Triumph, are designated as S and Mo-S and the male-fertile revertant WPR-1 line as F. Mitochondrial RNAs were isolated from etiolated seedlings, denatured with glyoxal, separated in an agarose gel, blotted onto nylon membranes, and hybridized with radiolabeled probes. Positions and sizes of the CMS-unique *pvs* transcripts are indicated at right in kilobases, and those of the *atpA/orf209* are at left in kilobases.

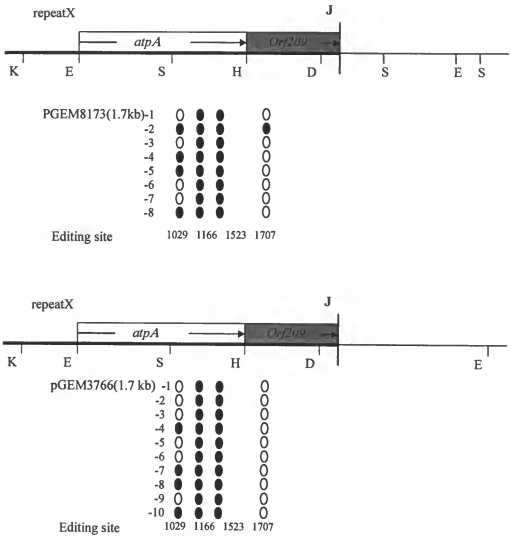

Fig. 1-3. Diagrammatic representation of RNA editing in the *atpA/orf209* region. The map of the *atpA/orf209* region was enlarged from the Fig 1-1. The filled ovals indicate the edited sites, and the open ovals unedited sites. Numbering of edited sites is as shown in Fig. 1-4.

Fig. 1-4. Nucleotide sequences and RNA editing sites in the *atpA/orf209* segment of *P. vulgaris*. The nucleotide sequence of *atpA* and *orf209* is shown as upper case letters. The predicted amino-acid residues are given under the DNA nucleotides. Untranslated non-coding sequences are shown as lower case letters. The editing sites are bold C indicated by the filled triangles (black-complete editing; gray-partial editing) in boxes. Corresponding amino-acid changes are given below by arrow. Numbering of nucleotides shown at the left side begins from the first base pair of the predicted translation start codon of *atpA* gene.

```
-100  tatgctatgatggaaaataggatatgtgggtttcagattctttctctaggccctttttgtaaaggcattcccccctgttatagaaggtcaaaagataaatt

1     ATG GAA TTC TCT TCC AGA GCT GCG GAA CTA ACC ACT CTA TTA GAA AGT AGA ATG ACC AAC
      M  E  F  S  S  R  A  A  E  L  T  T  L  L  E  S  R  M  T  N
         ⟶ atpA

61    TTT TAC ACA AAT TTT CAA GTG GAT GAG ATC GGT CGA GTG GTC TCC GTT GGA GAT GGG ATT
      F  Y  T  N  F  Q  V  D  E  I  G  R  V  V  S  V  G  D  G  I

121   GCA CGT GTT TAT GGA TTG AAC GAG ATT CAA GCT GGG GAA ATG GTT GAA TTT GCC AGC GGT
      A  R  V  Y  G  L  N  E  I  Q  A  G  E  M  V  E  F  A  S  G

181   GTG AAA GGA ATA GCG TTG AAT CTT GAG AAT GAG AAT GTC GGA ATT GTG GTC TTT GGT AGT
      V  K  G  I  A  L  N  L  E  N  E  N  V  G  I  V  V  F  G  S

241   GAT ACC GCT ATA AAA GAA GGA GAT CTT GTC AAA CGC ACT GGA TCT ATT GTG GAT GTT CCT
      D  T  A  I  K  E  G  D  L  V  K  R  T  G  S  I  V  D  V  P

301   GCG GGA AAG GCT ATC GTA GGG CGT GTG GTC GAC GCG TTG GGA GTA CCT ATT GAT GGA AGA
      A  G  K  A  M  L  G  R  V  V  D  A  L  G  V  P  I  D  G  R

361   GGG GCT CTA AGC GAT CAC GAG CGA AGA CGT GTC GAA GTG AAA GCC CCT GGT ATT ATT GAA
      G  A  L  S  D  H  E  R  R  R  V  E  V  K  A  P  G  I  I  E

421   CGT AAA TCT GTG CAC GAG CCT ATG CAA ACA GGG TTA AAA GCG GTA GAT AGC CTG GTT CCT
      R  K  S  V  H  E  P  M  Q  T  G  L  K  A  V  D  S  L  V  P

481   ATA GGC CGT GGT CAA CGA GAA CTT ATA ATC GGG GAC CGA CAA ACT GGA AAA ACA GCT ATT
      I  G  R  G  Q  R  E  L  I  I  G  D  R  Q  T  G  K  T  A  I

541   GCT ATC GAT ACC ATA TTA AAT CAA AAG CAA ATG AAC TCA AGG GCC ACC TCA GAG AGT GAG
      A  I  D  T  I  L  N  Q  K  Q  M  N  S  R  A  T  S  E  S  E

601   ACA TTG TAT TGT GTC TAT GTA GCG ATT GGA CAG AAA CGC TCA ACT GTG GCA CAA TTA GTT
      T  L  Y  C  V  Y  V  A  I  G  Q  K  R  S  T  V  A  Q  L  V

661   CAA ATT CTT TCA GAA GCG AAT GCT TTA GAA TAT TCC ATT CTT GTA GCA GCC ACC GCT TCG
      Q  I  L  S  E  A  N  A  L  E  Y  S  I  L  V  A  A  T  A  S

721   GAT CCT GCA CCT CTG CAA TTT CTG GCC CCA TAT TCT GGG TGT GCC ATG GGG GAA TAT TTC
      D  P  A  P  L  Q  F  L  A  P  Y  S  G  C  A  M  G  E  Y  F

781   CGC GAT AAT GGA ATG CAC GCA TTA ATA ATC TAT GAT GAT CTT AGT AAA CAG GCC GTG GCA
      R  D  N  G  M  H  A  L  I  I  Y  D  D  L  S  K  Q  A  V  A

841   TAT CGA CAA ATG TCA TTA TTG TTA CGC CGA CCA CCA GGC CGT GAG GCT TTC CCA GGC GAT
      Y  R  Q  M  S  L  L  L  R  R  P  P  G  R  E  A  F  P  G  D

901   GTT TTC TAT TTA CAT TCC CGT CTC TTA GAA AGA GCC GCT AAA CGA TCG GAC CAG ACA GGT
      V  F  Y  L  H  S  R  L  L  E  R  A  A  K  R  S  D  Q  T  G

961   GCA GGT AGC TTG ACC GCC TTA CCC GTC ATT GAA ACA CAA GCG GGA GAC GTA TCG GCC TAT
      A  G  S  L  T  A  L  P  V  I  E  T  Q  A  G  D  V  S  A  Y

1021  ATT CCA ACC AAT GTG ATC TCC ATT ACT GAT GGA CAA ATA TGT TTG GAA ACA GAG CTC TTT
      I  P  T>T  N  V  I  S  I  T  D  G  Q  I  C  L  E  T  E  L  F

1081  TAT CGC GGA ATT AGA CCT GCT ATT AAC GTC GGC TTA TCT GTC AGT CGC GTC GGG TCT GCC
      Y  R  G  I  R  P  A  I  N  V  G  L  S  V  S  R  V  G  S  A

1141  GCT CAG TTG AAA GCT ATG AAA CAA GCC TGC GGT AGT TTA AAA CTG GAA TTG GCA CAA TAT
      A  Q  L  K  A  M  K  Q  A>V  C  G  S  L  K  L  E  L  A  Q  Y

1201  CGC GAA GTC GCC GCC TTT GCT CAA TTT GGC TCA GAC CTT GAT GCT GCG ACT CAG GCA TTA
      R  E  V  A  A  F  A  Q  F  G  S  D  L  D  A  A  T  Q  A  L
```

```
1261  CTC AAT AGA GGT GCA AGG CTT ACA GAA GTA CTG AAA CAA CCA CAA TAT GCA CCA CTT CCA
      L   N   R   G   A   R   L   T   E   V   L   K   Q   P   Q   Y   A   P   L   P

1321  ATT GAA AAA CAA ATT CTA GTC ATT TAT GCA GCT GTC AAT GGA TTC TGT GAT CGA ATG CCA
      I   E   K   Q   I   L   V   I   Y   A   A   V   N   G   F   C   D   R   M   P

1381  TTA GAT AAA ATT CCT CAA TAT GAA AGA GAC ATT CTA ACG ACT ATT AAA CCT GAA TTA CTA
      L   D   K   I   P   Q   Y   E   R   D   I   L   T   T   I   K   P   E   L   L

1441  CAA TCA CTA AAA GGT GGA CTA ACT AGC GAA AGA AAA ATA GAA CTA GAA AAA TTC TTA AAA
      Q   S   L   K   G   G   L   T   S   E   R   K   I   E   L   E   K   F   L   K

1501  GAA AAA GCA AAA AAT TAC ACATT A TG A AA AAA AAG CTT ATG ATG AAA GAT AGG ATT TTA
      E   K   A   K   N   Y   T>I L     *
                                M   K   K   K   L   M   M   K   D   R   I   L
                                    orf209

1560  CGA GGA TTC TTT TTT ACT CTC TTG CTT TGT ATG TTA AAA AAT GGA ATG TAT TCG TTG GTA
      R   G   F   F   F   T   L   L   L   C   M   L   K   N   G   M   Y   S   L   V

1620  TCT GAT TTT GAT TTA TAT ACC ATC CTT GCT TGG TGG ATT CCC ACC TTT TAT ATG GAC GAC
      S   D   F   D   L   Y   T   I   L   A   W   W   I   P   T   F   Y   M   D   D

1680  GTG GGC GCC AGC TCA CAG TCG TCC TTG CCC CCT GCA GAG CCT CAA GCC CCT GAG CCC TTG
      V   G   A   S   S   Q   S   S   L   P>S P   A   E   P   Q   A   P   E   P   L

1740  CCC CCT GCA GAG CCT CAA GCC CCT GAC CTT TCC CAT ACT TTA CTG GAT GAC AAC ACT CGT
      P   P   A   E   P   Q   A   P   D   L   S   H   T   L   L   D   D   N   T   R

1800  CGC GCC GAA CTA GAT GAG CGC GCG GGG TTT CAT TTT GTG GGG TTG TCG GAA GAG AAG AAA
      R   A   E   L   D   E   R   A   G   F   H   F   V   G   L   S   E   E   K   K

1860  GAA AAA GTG CTT TTG GCA CAA GTC CAA ATA GAA AGG GCC ATT GAG AAA GCT CTG CTC TCC
      E   K   V   L   L   A   Q   V   Q   I   E   R   A   I   E   K   A   L   L   S

1920  GAC GGG TAT TCC CGG GAT GAG CTT TCT CAA AGG AGC AAA CGA GAT GAG ATT AGG GGC TTT
      D   G   Y   S   R   D   E   L   S   Q   R   S   K   R   D   E   I   R   G   F

1980  TTG TTC TAC CGT AAT GGG AAA CTT CTT TCT ATT AAG AAA TAC GAG TCT TAT GTG GAA GAA
      L   F   Y   R   N   G   K   L   L   S   I   K   K   Y   E   S   Y   V   E   E

2040  GTC AAA TTT GGG ACC CAC CGC AGC CAG CCA TAT CAA GAT CTT ATC CAT GCC ATC TCT GAT
      V   K   F   G   T   H   R   S   Q   P   Y   Q   D   L   I   H   A   I   S   D

2100  TCC TAT CTT TTT TTA AAG AAA GTA AAA AAA ATA AAG AGG TGG GAA CGG GGG TGA
      S   Y   L   F   L   K   K   V   K   K   I   K   R   W   E   R   G   *

2155  aacaga ttgggctcacagaatacttcagggctggccacgcacctgtcaggaactgtataaaaaaggaccgagaactacataaggtctgagacg
                pvs
2248  gacttccttgaagggagtctagttatgtaggcagtggccgttccggttgctcgaccaaagccggtcaataagacagatccggatcctagtgctttc
2344  gagatggttcgatcgctaacaaagacaagcatgggaaacagcagactcccgcaactccaagagctcaagcctaaagccttagtaacgcgcatcc
2538  tcattgctcgcgtaaagcaagcgtataaggtttattgatggacattcctatagaaactatttcccgcttggcagtagttgttt
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(Fig. 1-4 continued)

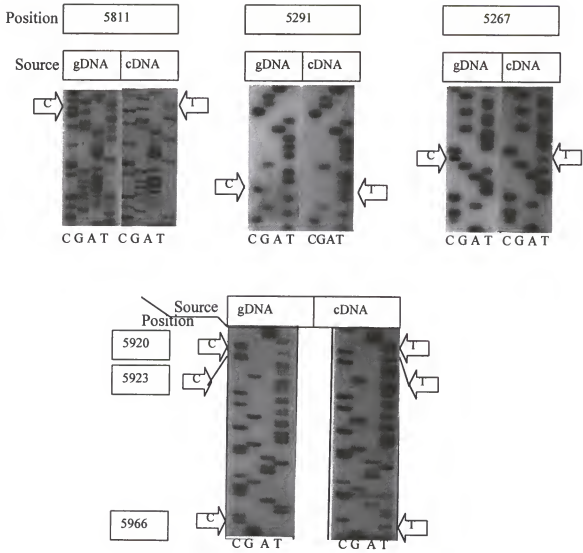


Fig. 1-5. Autoradiographs of sequencing gels for comparison of the mitochondrial genomic DNA (gDNA) and cDNA in the *orf239* and 3' *cob* segment of *P. vulgaris*. Edit positions and source of template DNAs are shown in the upper boxes. The positions indicate nucleotides numbered as shown in Fig. 1-4.

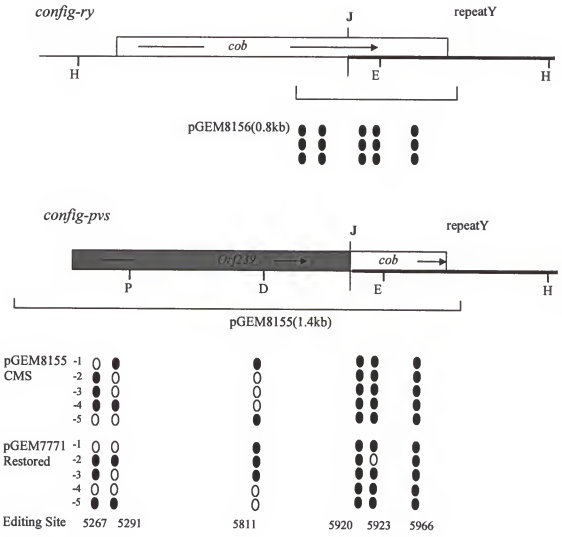
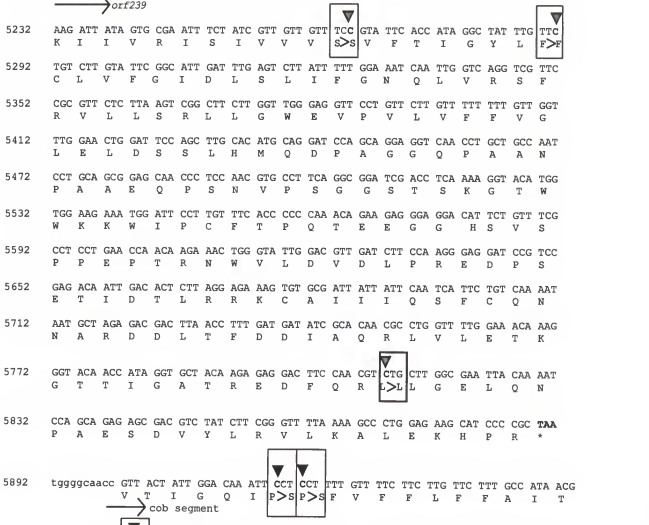


Fig. 1-6. Diagrammatic representation of RNA editing in the *orf239/cob* segment. The map of the *orf239/cob* segment was enlarged from the Fig 1-1. The filled ovals indicate the edited sites, and the open ovals the un-edited sites. Numbering of edited sites begins at the first base pair of the predicted translation start codon of *atpA* gene, located upstream of the *pvs* region.

Fig. 1-7. Nucleotide sequences and RNA editing sites in the *orf239/cob* segment. The complete sequences of *orf239* and the 3' *cob* segment are shown as upper case letters. The predicted amino-acid residues are given under the DNA nucleotides. Untranslated non-coding sequences are shown as lower case letters. The editing sites are bold C indicated by the filled triangle (black-complete editing; gray-partial editing) in boxes. Corresponding amino-acid changes are given below by arrow. Numbering of nucleotides shown on the left side starts from the first base pair of the predicted translation start codon in the *atpA* gene.


5172
ATG TTC CTC CCA TTC AAC CCA CAA AAA ATT TTC GGA AGA TTG AGT TCA ACT CTG TCG CGA
M F L P F N P Q K I F G R L S S T L S R
orf239
5232
AAG ATT ATA GTG CGA ATT TCT ATC GTT GTT GTT TCC GTA TTC ACC ATA GGC TAT TTG TTC
K I I V R I S I V V V S>S V F T I G Y L F>F
5292
TGT CTT GTA TTC GGC ATT GAT TTG AGT CTT ATT TTT GGA AAT CAA TTG GTC AGG TCG TTC
C L V F G I D L S L I F G N Q L V R S F
5352
CGC GTT CTC TTA AGT CGG CTT CTT GGT TGG GAG GTT CCT GTT CTT GTT TTT TTT GTT GGT
R V L L S R L L G W E V P V L V F F V G
5412
TTG GAA CTG GAT TCC AGC TTG CAC ATG CAG GAT CCA GCA GGA GGT CAA CCT GCT GCC AAT
L E L D S S L H M Q D P A G G Q P A A N
5472
CCT GCA GCG GAG CAA CCC TCC AAC GTG CCT TCA GGC GGA TCG ACC TCA AAA GGT ACA TGG
P A A E Q P S N V P S G G S T S K G T W
5532
TGG AAG AAA TGG ATT CCT TGT TTC ACC CCC CAA ACA GAA GAG GGA GGA CAT TCT GTT TCG
W K K W I P C F T P Q T E E G G H S V S
5592
CCT CCT GAA CCA ACA AGA AAC TGG GTA TTG GAC GTT GAT CTT CCA AGG GAG GAT CCG TCC
P P E P T R N W V L D V D L P R E D P S
5652
GAG ACA ATT GAC ACT CTT AGG AGA AAG TGT GCG ATT ATT ATT CAA TCA TTC TGT CAA AAT
E T I D T L R R K C A I I I Q S F C Q N
5712
AAT GCT AGA GAC GAC TTA ACC TTT GAT GAT ATC GCA CAA CGC CTG GTT TTG GAA ACA AAG
N A R D D L T F D D I A Q R L V L E T K
5772
GGT ACA ACC ATA GGT GCT ACA AGA GAG GAC TTC CAA CGT CTG CTT GGC GAA TTA CAA AAT
G T T I G A T R E D F Q R L>L L G E L Q N
5832
CCA GCA GAG AGC GAC GTC TAT CTT CGG GTT TTA AAA GCC CTG GAG AAG CAT CCC CGC TAA
P A E S D V Y L R V L K A L E K H P R *
5892
tggggcaacc GTT ACT ATT GGA CAA ATT CCT CCT TTT GTT TTC TTC TTG TTC TTT GCC ATA ACG
V T I G Q I P>S P>S F V F F L F F A I T
cob segment
5956
CCC ATT ATT CCG GGA CGA GTT GGA AGA GGA ATT CCT AAT TCT TAC ACT ACG GAT GAG ACT
P I I P>L G R V G R G I P N S Y T T D E T
6016
TAG atgtgaaaaattctgacaccaatcatttacgtattacaccaagaatgaattgacaagcgcattcg
*

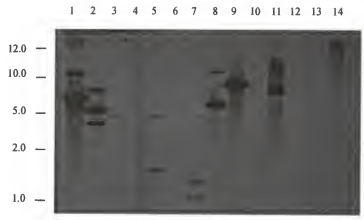


Fig. 1-8. Southern blot hybridization assay of total DNA with the *orf239* probe. Total DNAs from (1) CMS-Sprite common-bean, (2) soy-bean, (3) pea, (4) cow-pea, (5) broad-bean, (6) lupines, (7) crimson-clover, (8) peanut, (9) watermelon, (10) tomato, (11) tobacco, (12) petunia, (13) *Arabidopsis,* and (14) turnip, were digested with *Pst*I, fractionated by agarose gel electrophoresis, transferred to nylon supports, and probed with a radiolabeled *orf239* specific probe. The sizes of the restriction bands are indicated in kilobases.

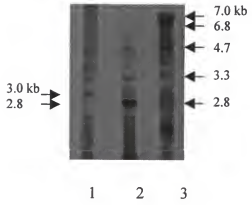


Fig. 1-9. RNA gel blot analysis with the *orf239* probe in soybean and broad bean. The mtRNAs from (1); broad-bean, (2); soybean, (3); common-bean were isolated from etiolated seedlings, denatured with glyoxal, separated in an agarose gel, blotted onto nylon membrane, and hybridized with radiolabeled *orf239* specific probe. The sizes of relevant transcripts are indicated in kilobases.

Table 1-4. Comparison of the *atpA* RNA editing between common bean and sugar beet.

Editing Site	Common-bean		Sugar-beet*	
	Codon Change	Amino acid	Codon Change	Amino acid
Site 1029	acC->acT	T -> T	ACC	T
1166	gCc->gTc	A -> V	GTC	V
1523	aCa->aTa	T -> I	TAA	Stop
1178	TTA	L	cCa->cTa	P -> L
1292	CTG	L	cCg->cTg	P -> L
1415	CTA	L	cCa->cTa	P -> L

*: Senda et al., 1993.

Table 1-5. Comparison of the editing sites found in a *cob* gene segment among different plant species.

Species \ Editing site*		Site 5920	Site 5923	Site 5966	Source
Common-bean	Codon	Cct->Tct	Cct->Tct	cCg->cTg	this study
	Amino acid	P -> S	P -> S	P -> L	
Potato	Codon	TCT	Cct->Tct	CTG	Zanlungo et al., 1993
	Amino acid	S	P -> S	L	
T. aestivum	Codon	Cct->Tct	TCT	cCg->cTg	Zanlungo et al., 1993
	Amino acid	P -> S	S	P -> L	
Oenothra	Codon	TCT	Cct->Tct	CTG	Zanlungo et al., 1993
	Amino acid	S	P -> S	L	

*: Number based on Chase and Ortega, 1992

Table 1-6. Frequency of silent editing in plant mitochondria.

Species	Gene	Position	Editing Freq.	Codon Change	Amino acid	Source
Common bean	*orf239*	5267	6/10	tcC-> tcT	Ser	present study
		5291	3/10	ttC-> ttT	Phe	
		5811	5/10	Ctg-> Ttg	Leu	
	atpA	1029	2/5	acC->acT	The	
Maize	*mat-r*	806	3/14	ctC-> ctT	Leu	Thomson et al., 1994
		2558	2/9	taC-> taT	Y	
		2678	8/10	gtC-> gtT	Val	
Oenothera	*cob*	8th	2/7	ttC->ttT	Phe	Schuster et al., 1991
		13th	3/8	ctC->ctT	Leu	
	atpA	2nd	6/18	acC->acT	The	
		6th	8/10	Cta->Tta	Leu	
Sorghum	*atp6*	363	5/13	acC->acT	The	Kempken et al., 1991
		477	2/13	ctC->ctT	Leu	
		483	8/13	tCg-> tTg	Leu	
	atp9	220	17/20	Ctg-> Ttg	Leu	Salazar et al., 1991
Sugar beet	*orfB*	30	2/6	ttC->ttT	Phe	Kubo & Mikami, 1996
Sunflower	*nad3*	102	partial	acC->acT	The	Perrotta et al., 1996

CHAPTER 2: IDENTIFICATION OF A LOW-ABUNDANT DNA MOLECULE ASSOCIATED WITH CMS IN *PHASEOLUS VULGARIS.*

Literature Review

Mitochondrial Genomes in Higher Plants

The mitochondrial genomes of higher plants (reviewed by Fauron, 1995a,b; Pring and Lonsdale, 1985; Bendich, 1993; Schuster and Brennicke, 1994) are not only large in genome size, but also are variable in their organization, compared with their animal or fungal counterparts (reviewed by Shadel and Clayton, 1997; Attardi, 1988). Their size from 208 kb of *Brassica hirta* (Palmer and Hebron, 1987) to approximately 2,500 kb of muskmelon (Ward et al., 1981) greatly exceeds the amount of DNA necessary to account for the coding functions (Fauron et al., 1995a; Unseld et al., 1997). Recently the complete genome (367 kb) of the mitochondrial DNA (mtDNA) in the *Arabidopsis thaliana* has been sequenced (Unseld et al., 1997), and only 10% of the genome encodes 57 identified genes. The function of 60% of the genome is unknown.

The plant mitochondrial genome has the ability to undergo homologous recombination between repeats that vary in size from 6 bp up to 14 kb sequences (Lonsdale et al., 1984; Palmer and Shields, 1984; reviewed by Andre et al., 1992). Recombination at repeated regions has been suggested to generate the mutipartite organization of the maize mitochondrial genome (Lonsdale et al., 1984). A very similar mechanism is thought to provide a specific recombination site for the interconversion of three circular mtDNA molecules in *Brassica* species (Palmer and Shields, 1984). In *Brassica* species, there is one major repeat and more than 50 small repeats that fall into 10 or more families. However, *Brassica hirta* (208kb master circle map) is an exception lacking large repeats (Palmer and Hebron, 1987 and reviewed by Andre et al., 1992). The *in vivo* genome of *Brassica hirta* is believed to exist as a single master circle (Palmer and Hebron, 1987). Rottmann et al., (1987) and Fauron et al. (1990a,b) provided direct evidence indicating that homologous recombination takes place during reversion of CMS-T maize to male fertility. Homologous recombination takes place between two sets of repeated sequences (Rottmann et al., 1987 and Fauron et al., 1990a,b). The *urf13* gene in the T male-sterile cytoplasm of maize is lost via homologous recombination during reversion to fertility (Rottmann et al., 1987). Finally, recombination of plant mtDNA has been

shown in fusion of somatic cells with genetically different cytoplasms, resulting in non-parental restriction fragments with the resulting hybrids (Belliard et al., 1979).

Generation of Sub-stoichiometric Molecules through Recombination

Due to recombination at repeated sequences, the plant mitochondrial genome could exist in many different configurations, albeit at varying stoichiometry (Small et al., 1989). Variation in the relative stoichiometry of mtDNA configurations has been reported and shown to be widespread in plant cytoplasms (Morgens et al., 1984; Small et al., 1987). More than five repeat sequences active in recombination have been identified in maize cytoplasms including Texas (T) cytoplasm, giving rise to various isomeric forms and subgenomic DNA molecules of different stoichiometry (Fauron et al., 1990b; Lonsdale et al., 1984). Four genomic configurations containing the *atpA* gene were previously considered to be unique to male-sterile S and T cytoplasms of maize, but those configurations are actually present in low abundance in fertile cytoplasms (Small et al., 1987). Sub-stoichiometric molecules present in one or both of the fusion parents were amplified in somatic hybrids of the *Gramineae* (Ozias-Akins et al., 1988).

Most of the mtDNA configurations are generated by recombination through large repeats. However, the low-abundant, sub-stoichiometric molecules termed 'sublimons' (Small et al., 1987) have been known to be generated by infrequent recombination between small repeats (6-689 bp) (reviewed by Andre et al., 1992) according to the recombination model suggested by Small et al. (1989). Small et al. (1989) also suggested that nuclear gene changes could alter the relative copy number of the various mitochondrial genomes. These sublimon molecules must be maintained by normal replication or are continuously being generated by *de novo* recombination since sublimon is retained in the genome for many generations at low level (Small et al., 1987).

Physical Structure of the Mitochondrial DNA

A "master circle" has been proposed for the genome structure on the basis of physical mapping from *Brassica campestris* (Palmer and Shields, 1984) and *Zea mays* (Lonsdale et al., 1984). The high frequency of recombination between pairs of repeat sequences on a "master circle" leads to mtDNA rearrangement and duplication to contribute to mitochondrial genome expansion in plants (Fauron et al., 1990; Palmer and Herbon, 1986). More than one autonomous molecule was suggested for the structure of the

plant mitochondrial genome in some species (Folkerts and Hanson, 1991; Levy et al., 1991; Janska and Mackenzie, 1993). However, recent studies using pulsed field gel electrophoresis and moving picture analysis suggested that physical structures of intact mitochondrial genomes in plants are mainly large linear and branched molecules. Circular mtDNAs smaller than genome size have been observed, but are always rare (Oldenburg and Bendich, 1996; Backert et al., 1995; Bendich and Smith, 1990; Bendich, 1996). Only small amounts of open circles and sigma-like molecules are detected in plant mitochondrial genomes (Bendich, 1993; Backert et al., 1996). Thus, the *in vivo* structure of the large mitochondrial genome and the existence of a master molecule have not been clearly demonstrated.

In study of cultured tobacco cells, pulse-chase experiments with ^{3}H-thymidine suggested that the newly synthesized mtDNA is predominantly in the well-bound fraction. Most of well-bound mtDNA is present in branched structure of multigenomic concatemers larger than the 270 kb size of the tobacco mitochondrial genome (Oldenburg and Bendich, 1996). Thus, they suggested that mtDNA replication in plants was initiated by recombination since most of the newly synthesized mtDNA was in branched forms of multigenomic concatemers.

Gene Contents and Nuclear Influences in Plant Mitochondria

The number of genes encoded in plant mitochondria differs among plant species. In general, mitochondrial genomes encode ribosomal RNA (rRNA) (18/5S and 26S), several transfer RNA's (tRNA) (10 potential tRNA genes in maize; 25 in yeast), ribosomal proteins (10 to 16 small and large subunit), NADH dehydrogenase complex (*nad1* to *nad9*), cytochrome c oxidase complex (*cox*I, II, and III), ATPase complex (6, 9, and α), and the apocytochrome b (*cob*) (reviewed by Schuster and Brennicke, 1994; Newton, 1988). Additional open reading frames have been identified, and therefore, it is possible that more of the functional genes are present in plant mitochondria. In higher plants, the 18S and 5S genes are closely linked (Chao et al., 1983).

Plant mitochondrial genomes do not contain a complete set of genes encoding the tRNAs (Unseld et al., 1997). Additional tRNAs for a complete coding system are imported from the cytosol (Marechal-Drouard et al., 1988). There is a evidence showing that a few mitochondrially encoded tRNAs are of chloroplast origin (Joyce and Gray, 1989). Therefore, the mtDNA maintains a small number of protein-coding genes as well as rRNAs and tRNAs, and most other mitochondrial proteins are nuclearly encoded and imported post-translationally into the mitochondria.

Transcripts of the plant mitochondrial genes are often large with multiple transcript species for a particular coding region (reviewed by Newton, 1988). RNA processing and multiple transcription initiation sites result in multiple transcript sizes in maize mitochondria (Mulligan et al., 1988). Further studies are necessary to examine how the large multipartite genome of mitochondria contributes to transcriptional regulation.

Nuclear gene products regulate the mitochondrial genomes and their gene expression (He et al., 1995; reviewed by Hanson and Conde, 1985). Mitochondrial genomes can also influence nuclear gene expression in yeast (Liao and Butow, 1993). These observations demonstrate that nuclear and mitochondrial genes coordinately interact with each other for proper cellular functions (Forsburg and Guarente, 1989). One of the best examples of nuclear gene influence on mitochondrial genomes is a nuclear restorer of the CMS system in *Phaseolus vulgaris*. A nuclear fertility restorer gene designated *Fr* irreversibly alters the mitochondrial genome structure by eliminating the CMS-unique sequence (Mackenzie et al., 1988). In *Arabidopsis*, the nuclear chloroplast mutator (CHM) locus results in mtDNA rearrangements (Sakamoto et al., 1996). These mtDNA rearrangements result from the amplification of sublimon molecules generated by homologous recombination between short repeats.

CMS

The mitochondria of higher plants are not only the major sites of energy production by oxidative phosphorylation (reviewed by Attardi, 1988), but also have genes associated with the CMS phenotype (reviewed by Dewey et al., 1986; Hanson, 1991). CMS, the failure to produce functional pollen during microsporogenesis, is inherited through the mitochondria of female parent (Rhoades, 1950). The CMS phenotype results from the expression of novel chimeric genes in the mitochondria (reviewed by Hanson, 1991) and can be suppressed by nuclear restorer-of-fertility genes (reviewed by Hanson and Conde, 1985). In most cases, CMS plants are morphologically normal other than pollen abortion due to floral-specific aberrations (Laser and Lersten, 1972).

In *Phaseolus vulgaris*, mtDNA (mtDNA) sequences (3,736 bp) designated *pvs* (for ***Phaseolus vulgaris*** sterility) are associated with CMS (Chase and Ortega, 1992; Johns et al., 1992). At least two different nuclear fertility restoration systems have been identified (Mackenzie et al., 1988; Mackenzie, 1991). The nuclear fertility restorer gene *Fr* irreversibly alters the mitochondrial genome as mentioned above (Mackenzie et al., 1988), while another fertility restorer, *Fr2,* does not alter the mitochondrial genome so that the *pvs* sequence is present in the *Fr2* restored line (G08063)

(Mackenzie and Chase, 1990; Mackenzie, 1991). As *pvs* is lost upon spontaneous reversion to fertility, the *pvs* region is absent in the spontaneous revertant line (WPR-1) as well as the *Fr*-restored line (Johns et al., 1992; Mackenzie and Chase, 1990; Mackenzie et al., 1988).

The *pvs* and related mitochondrial transcripts have been characterized by northern analysis (Mackenzie and Chase, 1990; Chase, 1994). Northern hybridization revealed CMS-unique *pvs* transcripts of 7.0, 6.8, 4.7, 3.3, and 2.8 kb. A 7.0 kb RNA is the most abundant transcript, and sedimentation assays indicated that the 7.0 kb transcript is associated with polyribosomes (Chase, 1994). A 2.1 kb segment of the 7.0 kb transcript is not accounted for by northern hybridizations with the *pvs* or flanking DNA clones (Chase, 1994). It was suggested that RNA splicing processes and/or aberrant DNA configurations (e.g. sublimon) could explain the origin of the unidentified 2.1 kb in the generation of the CMS-unique 7.0 kb transcript (Chase, 1994). Identification of this 2.1 kb segment and characterization of its sequence is a major objective for understanding expression of *pvs* encoded gene products associated with CMS.

The *pvs* region is flanked by two different repeat sequences x and y in a configuration we designate the *config-pvs* (Fig. 2-1). Configurations designated config-rx and the config-ry contain other copies of repeat X or

repeat Y, respectively. In the *pvs* the longest open reading frame is *orf239*, predicting a protein of 239 amino acids and unknown origin. The repeat x includes a complete copy of the F1 ATPase subunit A (*atpA*) gene and an overlapping open reading frame (*orf209*). An apocytochrome b (*cob*) segment (117 nt) is located in repeat y. The predicted translation product of *orf209* shared 69% identity with its soybean counterpart *orf214*.
The organization of the *orf209* and *atpA* is similar between soybean and common bean (Chase and Ortega, 1992; Chanut et al., 1993). In addition, Covello and Gray, (1992) suggested that common bean and soybean are closely related species based upon *coxII* gene transfer from mitochondria to the nucleus.

To determine the location of the unidentified 2.1 kb segment on the 7.0 kb transcript, oligonucleotide-directed RNase H digestion assays were performed with mitochondrial RNA (mtRNA) of CMS-Sprite. This experiment indicated that physical structure of the 7.0 kb *pvs* RNA was linear and that the unidentified 2.1 kb was located 5' of the *pvs* sequence. RNA splicing process or a sub-stoichiometric DNA template ('sublimon') could be the origin of the unidentified 2.1 kb in the generation of the CMS-unique 7.0 kb transcript. I investigated origin of this transcript and report

that a mtDNA configuration containing the 18S/5S rRNA genes and *pvs* sequences was present as a sublimon and transcribed in CMS lines.

Materials and Methods

Plant Material

The *P. vulgaris* lines used in this work {CMS-Sprite (a male-sterile line), G08063 (a male-fertile line restored by *Fr2*), Mo-triumph (a male-sterile line) and WPR-1 (a male-fertile cytoplasmic revertant derived from the CMS-Sprite)} are described in Table 2-1. The mtDNA configurations characteristic of these lines are shown in Fig. 2-1.

Table 2-1. Genetic Materials

Line	Phenotype	MtDNA Configurations	Nucleus	Source
CMS-Sprite	male-sterile	*rx, ry and pvs*	non-restoring *fr2/fr2*	Bassett & Shuh, 1982.
WPR-1	male-fertile	*rx and ry*	non-restoring *fr2/fr2*	Mackenzie et al., 1988.
G08063	male-fertile	*rx, ry and pvs*	restoring *Fr2/Fr2*	Mackenzie, 1991.
Mo-triumph	male-sterile	*pvs and ry*	non-restoring *fr2/fr2*	Hervieu et al., 1993

Isolation of Mitochondria and Extraction of Nucleic Acids

Mitochondria were purified by differential centrifugation from 5-7 day old etiolated cotyledons and hypocotyls and lysed in the presence of SDS. MtDNAs were purified from the isolated mitochondria as described by Wilson et al. (1989). For the preparation of mtRNA, mitochondria were lysed in 6M guanidine thiocyanate; RNA was purified by extraction with phenol and chloroform, followed by precipitation with isoamylalcohol as described by Chase (1994).

Gel Electrophoresis, Nucleic Acid Transfer, and Blot Hybridization

DNA samples were digested with restriction endonucleases, fractionated by agrose gel electrophoresis and transferred to nylon supports (Hybond-N, Amersham Corp.) as described by Wilson et al. (1989). The fractionated DNA was transferred to nylon membranes by capillary blotting (Sambrook et al., 1989) and cross-linked by baking (1h at 80°C). The mtRNAs were denatured in glyoxal, separated by agarose gel electrophoresis, and transferred to a nylon membrane as described by Chase (1994). Southern and Northern blot hybridization experiments were carried out by the methods described in Chase, (1994) except for modified buffers and hybridization conditions. SSPE buffer (5X; 0.9 M NaCl, 50 mM

NaH_2PO4, and 5mM Na_2EDTA) and Denhart's solution (5X; 0.1% w/v BSA, 0.1% w/v Ficoll400, and 0.1% w/v PVP360) with 0.5% w/v SDS and 100 mg/ml sheared salmon sperm DNA were used for hybridization. SSPE buffer (0.1X) and 0.1% SDS were used for washing as described in the manufacture's protocol (Amersham Corp.). Hybridizing and washing temperatures were maintained at $60^{o}C$. The DNA template for labeling was purified from either PCR products or inserts recovered from cloned plasmid DNA. Templates were purified from agarose gels as described by Chase (1994). Purified DNA fragments were labeled by random priming (Feinberg and Vogelstein, 1984) with the RTS RadPrime DNA labeling system (Life Technologies) in the presence of [α-^{32}P]dCTP.

For transcription mapping, DNA probes were prepared from 17 cosmid library clones covering the 417 kb mitochondrial genome of the *P. vulgaris* cytoplasmic revertant line WPR-3 provided by S. Mackenzie. Each cosmid DNA (30–50 kb) was radiolabeled in the presence of [α-^{32}P]dCTP and the labeled probe was hybridized to the northern blots as described above.

PCR Amplification and Cloning.

A DNA fragment containing the 5' region of the 18S mitochondrial rRNA gene, 5S mitochondrial rRNA gene and the *pvs* region was amplified from mtDNA of *P. vulgaris* by PCR with primers described in Table 2-2. The thermocycler program of the PCR amplification was a pre-denaturation at 92°C for 2 min followed by 35 cycles of 92°C (35 s) for denaturation, 60°C (45 s) for annealing, and 72°C (1.5 min) for synthesis. The PCR products were purified from low-melting agarose (GTG) gels and then ligated into the pGEM-T plasmid vector (Promega Inc.) by utilizing the A-overhang of PCR products. The ligated DNAs were transformed into DH5α competent cells purchased from Life Technologies. Oligonucleotides (Table 2-2) specific to genes of interest were synthesized by the DNA Synthesis Core of the Interdisciplinary Center for Biotechnology Research at the University of Florida or as Custom Primers from Life Technologies.

Sequencing

Nucleotide sequences were obtained from double-stranded plasmid DNA sequenced on both strands by the dideoxy nucleotide chain-termination method (Sanger et al., 1977) with Sequenase version 2.0 (US Biochemicals). DNA polymerization was carried in the presence of [α-^{32}P]

dCTP by 1 unit of DNA polymerase Sequenase version 2.0. Samples were fractionated at 55 watts for 2 to 5 hours on 8% acrylamide/Urea gels in the 1X TBE and autoradiographed on Kodak diagnostic film X-Omat™ XRP-5.

RNase H Digestion Assay

The oligonucleotide-directed RNase H digestion experiment was carried out as described by Capel et al., (1993). Positions of annealed oligonucleotides and hybridizing probes of the RNase H digestion assay are shown in Fig. 2-1. Total mtRNA (5 μg) from CMS-Sprite was annealed with 10 ng of oligonucleotide 2, oligonucleotide 3, or oligonucleotide 4 at 37°C for 30 min in first-strand cDNA synthesis buffer from Life Technologies. The samples were treated with RNase H for 1 hr at 37°C, then denatured with glyoxal, separated by agarose-gel electrophoresis, and blotted to membrane supports. The ^{32}P-dCTP labeled DNA probes (592 bp probe F or 1038 bp probe H) derived from the *pvs* sequence were independently hybridized to the RNA blots. The construction of *P. vulgaris* mtDNA clones has been described by Chase (1994). The CMS-Sprite mtRNA without any treatment was used for control (lane C in Fig. 2-3). Sequences were numbered from the first nucleotide of the predicted initiation codon in the *atpA* gene located upstream of the *pvs* region (Chase

and Ortega, 1992). The positions of the oligonucleotide correspond to the first 5' nucleotides of each oligonucleotide.

Results

The Unidentified 2.1 kb Segment Was Located at the 5' End of the 7.0 kb *pvs* Transcript.

To determine the physical structure of the 7.0 kb *pvs* RNA molecule as well as the location of the unidentified 2.1 kb segment, oligonucleotide-directed RNase H cleavage assays were performed with mtRNA isolated from CMS-Sprite. The organization of CMS-unique and flanking mitochondrial DNA in CMS-Sprite and relative position of oligonucleotides for the RNase H cleavage assays are shown in Fig. 2-1. The extracted mtRNAs were annealed with specific oligonucleotides within the *pvs* region and digested with RNase H (Fig 2-2). RNase H digests RNA of RNA/DNA hybrids, resulting in a nick of the RNA molecule. The nicked RNA fragments were denatured with glyoxal, separated by agarose gel electrophoresis, immobilized to a membrane, and hybridized with radio-labeled DNA probes. If the *pvs* transcript exists as a linear molecule, then this treatment would result in two bands, which later could be detected on a northern blot with probes spanning the *pvs* region. On the other hand, if the

transcript is a circular molecule, oligonucleotide-directed RNase H digestion would yield only one band detectable on a northern blot. If the 7.0 kb RNA is an aberrant or lariat form, electrophoretic mobilities of the nick products would not be consistent with that of intact RNA (Capel et al, 1993; Backert et al., 1996). If the 7.0 kb RNA is linear, then the sizes of the two bands produced by oligonucleotide-directed nick would reveal the location of the unidentified 2.1 kb sequence relative to the 4.9 kb DNA region known to be co-linear with the 7.0 kb transcript. Thus, based on the numbers and sizes of the bands detected, we could deduce the physical structure and the location of the unidentified 2.1 kb region of the 7.0 kb *pvs* transcript.

Probe F hybridized to the northern blot containing the transcripts annealed by each of the three oligonucleotides (oligos 2, 3, and 4). The blot was reprobed with the 3' downstream probe H after the first probe decayed (Fig. 2-3). The resulting autoradiographs indicated that two fragments were generated with RNase H treatment in the presence of oligonucleotide 3 (position 2903), a 3.7 kb band identified by probe F (lane 3 of left panel) and a 3.3 kb band identified by probe H (lane 3 of the right panel). Another specific oligonucleotide 4 (position 4502) used independently gave similar results- a 5.3 kb fragment hybridized by probe F and a 1.7 kb fragment hybridized by probe H (lane 4). Without any treatment, the intact 7.0 kb *pvs*

transcript was detected by either probe F or H as a control (lane C). In the case of oligonucleotide 3, the sum of a 3.7 kb fragment detected by probe F and a 3.3 kb by probe H was consistent with the size of the targeted 7.0 kb *pvs* transcript as shown in diagrammatic representation (Fig. 2-3*B*). The same consistency was observed in case of oligonucleotide 4. Therefore, two fragments were derived from the 7.0 transcript and the sizes of the fragments generated by RNase H were dependent on the position of the specific oligonucleotides on the 7.0 kb *pvs* transcript. Moreover, in each experiment the electrophoretic mobilities of the resulting RNA fragments were consistent with a 7.0 kb *pvs* transcript. Therefore, the 7.0 kb *pvs* transcript was not a circular or sigma structure but a linear RNA molecule.

Concomitantly, the oligonucleotide-directed RNase H digestion assays enabled us to locate the unidentified 2.1 kb segment of the 7.0 kb *pvs* transcript. A notable observation in this experiment was that the sizes of the RNA fragments (the 3.3 kb from oligonucleotide 3 and the 1.7 kb from oligonucleotide 4) detected by probe H were consistent with the expected sizes if the 3' terminus of the 7.0 kb RNA was located in the region where colinearlity with the mtDNA ended. On the other hand, the 3.7 kb fragment from oligonucleotide 3 cleavage and the 5.4 kb fragment from oligonucleotide 4 cleavage detected by probe F were 2.1 kb larger than the

predicted sizes (1.6 kb and 3.3 kb, respectively) if the 5' terminus of the 7.0 kb transcript was located in the region where colinearity with the mtDNA ended. The 2.1 kb size increase was consistent with the size of the unidentified 2.1 kb segment. Therefore, the unidentified 2.1 kb segment was located to the region 5' of the *pvs* sequence on the 7.0 kb RNA. This 2.1 kb of the CMS-unique 7.0 kb RNA could be placed in the region of 5' of the *pvs* sequence by several mechanisms. One is by RNA splicing. Another is by transcription of an unknown DNA configuration, perhaps a sublimon.

5'RACE to Identify the Unidentified 2.1 kb of the *pvs* Transcript.

5'- rapid amplification of cDNA ends (RACE) initiated from primers within the *pvs* region was used to identify sequences (2.1 kb) located 5' of *pvs*. A 6.8 kb *pvs* transcript was previously shown to result from the co-transcription of *atpA* and the 3' downstream *pvs* sequences. RACE products were therefore expected to be from both transcripts (6.8 and 7.0 kb) when an antisense primer (oligo 2 in fig 2-1) within the *pvs* sequence was used for the synthesis of the first-strand cDNA. The cDNA amplified by PCR was cloned into the pGEM-T vector. Numerous 0.5-1.5 kb cDNA clones were recovered from the CMS-unique 6.8 *pvs* transcript that is co-linear with

DNA sequence, indicating that RACE technique worked for the synthesis of cDNA.

Three cDNA clones that were not co-linear with the DNA sequence were obtained from RACE. Upon sequencing, all three clones were found to contain an antisense 26S rRNA sequence as well as the *pvs* sequence and shared a common putative divergent site between 26S rRNA sequence and the *pvs* at position 1539. Northern blot hybridization and RT-PCR were performed in an attempt to verify these RACE products. As Northern blot hybridization with the 26S rRNA sequence as a probe could not verify that the identified 26S rRNA sequence was part of the 7.0 kb *pvs* transcript, we suspected that these RACE clones resulted from artifacts due to errors in the reverse transcription.

We suspected that RNA secondary structure prevented the recovery of bona fide RACE products from the 7.0 kb *pvs* transcript. Primer extension analysis was performed in the presence of the various RNA denaturants (DMSO, methyl-mercury, boiling followed by quick freezing) (Fig 2-4). The extended products were electrophoresed with a control DNA sequencing ladder generated on the *pvs* DNA clone. Although no transcript 5' termini were expected in the 3' segment of *orf209*, four major terminations were detected with somewhat reduced intensities compared with the nondenatured

treatment. Thus, we could assume that in the 7.0 kb *pvs* transcript secondary structure of the RNA was perhaps strong enough to prevent extension of polymerase for the 5'RACE. Therefore, an alternative approach, which was not dependent upon cDNA cloning for identification of the 2.1 kb segment of the 7.0 kb *pvs* transcript, was carried out.

Transcription Map

Previous study revealed the physical map of the 417 kb mitochondrial genome from the WPR-3 male-fertile revertant line (Janska and Mackenzie, 1993). They found that only the *pvs* sequence was unique to CMS-Sprite. In an effort to identify the unidentified 2.1 kb segment in the 7.0 kb *pvs* transcript, we performed Northern hybridization with 17 overlapping WPR-3 cosmid clones covering the entire WPR-3 mitochondrial genome. The cosmid library of mitochondrial clones from WPR-3 was developed previously in vector pWE15 (Stratagene) and the mitochondrial genes indicated on the map were placed by physical mapping with gene specific probes (Janska and Mackenzie, 1992). Northern blots containing mtRNAs isolated from CMS-Sprite and WPR-1 were hybridized with the radio-labeled WPR-3 cosmid clones. The autoradiographs are those of CMS-Sprite, to clearly demonstrate all of transcripts hybridized by each probe.

No difference was found between CMS-Sprite and WPR-1 transcription maps (Fig. 2-5).

Abundant and discrete transcripts were observed from 12 mitochondrial genes placed on the cosmid clones. Simple transcript patterns were observed from hybridization with cosmids known to carry *cox*III, *atp9,* and *atp6,* while complex transcript patterns (multiple transcripts) were revealed by hybridization with cosmids known to carry *cob, atpA, cox*II, *nad*I, and rRNA genes. A single abundant transcript of 1.4 kb was detected with clone 9D10 containing *atp6*, and a 1.6 kb transcript was detected with clone 3D8, which contains *cox*III. A 250 bp transcript of *atp9* was detected by four different clones (1G9, 10H9, 12D5, and 5AG) all previously shown to carry the *atp9* gene. As shown in previously published mtDNA maps (Janska and Mackenzie, 1992), two copies of *atpA* were identified in CMS-Sprite, whereas one was present in WPR-1. In this experiment, the size and abundance of the *atpA* transcripts appeared to be similar between these two lines (data not shown), suggesting that copy number of the genes is not correlated with the transcript abundance. Therefore, steady state level of transcript can be maintained at certain level regardless of the gain or loss of a transcribed gene configuration.

Mitochondrial transcript patterns were compared between a CMS line (CMS-Sprite) and a revertant line (WPR-1). Any cosmid clone which hybridizes to the CMS-unique 7.0 kb transcript could be a potential candidate clone containing the unidentified 2.1 kb segment of this RNA. We did not find any cosmid from the WPR-3 library that hybridized to the CMS-unique 7.0 kb transcript. The 7.0 kb transcript detected by cosmid clone 12C4 was found in CMS-Sprite as well as WPR-1, and was therefore not the CMS-unique 7.0 kb *pvs* transcript. The identical transcript patterns between CMS-Sprite and WPR-1 suggested that DNA sequence alterations of the reversion process did not affect transcription regulation.

A large number of transcripts were detected from uncharacterized mitochondrial DNA. These are probably transcripts of additional functional genes. This study demonstrated the dispersed distribution of transcription units in the approximately 417 kb mitochondria genome in *P. vulgaris*. The identity of additional functional genes in the mitochondria remains to be learned.

Identification of the Sublimon Configuration Containing the *pvs* Sequence.

Another possible explanation for the 7.0 kb RNA is that a mtDNA sublimon not observed in previous southern analysis was transcribed to give

rise to the 7.0 kb *pvs* transcript. Searches of the GenBank and EMBL database with FastA programs revealed sublimon configuration of 18S/5S rRNA genes fused to a 3' segment of *orf214* in soybean mitochondria (Morgens et al., 1984). Since Morgens et al. (1984) found a novel soybean mitochondrial transcript resulting from the sublimon mtDNA configuration involving a DNA rearrangement with the 5S rRNA gene and *orf214*, it was hypothsized that a sublimon molecule containing *18S/5S* and 3'region of *orf209* could be present in the mitochondrial genome of the common bean and be transcribed to give rise to the 7.0 kb pvs transcript.

Based upon the restriction map of the soybean 18S/5S and *orf214* DNA configuration, the predicted restriction map of the putative sublimon containing the common bean 18S/5S-*orf209-pvs* DNA region was generated. The predicted configuration was sought by southern blot hybridization analysis with 18S/5S rRNA and *pvs*-specific probes (Fig. 2-6). Since we were looking for a low-abundant, CMS-unique band, southern blots were exposed for a prolonged period of time for autoradiography. Screening *Pst*I-digested mtDNAs of CMS-Sprite and WPR-1 with a radio-labeled *pvs* probe detected one predominant 3.7 kb fragment in CMS-Sprite (see Figure 2-6, lane1) as expected from previous studies (Chase and Ortega, 1992). In addition, another *Pst*I restriction fragment of 5.5 kb was observed at low

levels in CMS-Sprite, but not in WPR-1. The size of this fragment was consistent with the predicted restriction map. The 5.5 kb band was reproducibly detected with different regions of the *pvs* sequence, suggesting that the 5.5 kb band represents a sub-stoichiometric molecule ('sublimon') containing the *pvs* sequence. Additional southern blot hybridization analysis with 18S/5S probes verified an *EcoR*I restriction fragment of 2.0 kb was uniquely present at low levels in the CMS-Sprite, but not in the WPR-1 (see Figure 2-6B, lane S). The presence of the 2.0 kb restriction fragment from the sublimon molecule was also consistent with the predicted restriction map (Fig. 2-6). Therefore, these results indicate the presence of the DNA organization containing *18S/5S* and the *pvs* sequence at some low level in the male-sterile cytoplasms.

Further confirmation of this DNA configuration was obtained by PCR. Primers based on *pvs* and soybean 18S/5S rRNA gene sequences were used to amplify a predicted 980 bp fragment (Fig. 2-7). The PCR product amplified from the putative sublimon was cloned into plasmid vectors, and sequenced. DNA cloning and sequencing of the 'sublimon' fragment demonstrated that the mitochondrial 18S/5S rRNA sequence was fused to the 3' portion of the *orf209* and the *pvs* sequence as predicted from Morgens et al. (1984) (Fig. 2-8). The point of divergence between the 18S/5S/*pvs*

configuration and the *atpA/orf209/pvs* configuration was at nucleotide 1851 in the latter configuration within *orf209* (Fig. 2-9). This configuration was confirmed by PCR with different sets of 18S/5S rRNA and *pvs* primers (Fig. 2-7). PCR products, 550 bp and 850 bp respectively, were amplified with primers (cc55 and cc101/cc102) as expected from CMS-Sprite and G08063, but not from revertant male-fertile WPR-1. PCR analysis of mtDNA from Mo-Triumph with the same primers verified the presence of the sublimon in this line as well. The presence of the sublimon molecule in CMS-inducing cytoplasms suggested that the sublimon molecule may be generated from the predominant form of the *config-pvs* by homologous recombination.

The MtDNA Sublimon Containing the *Pvs* Sequence Was Transcribed.

To determine if the sublimon molecule containing the 18S/5S rRNA genes and *pvs* was transcribed into the CMS-unique 7.0 kb transcript, RT-PCR amplification and northern hybridization analysis were performed. PCR amplification of reverse-transcribed cDNA synthesized from CMS-Sprite mtRNA with the primers (cc55/cc101) (Fig. 2-7) resulted in 550 bp fragment (see Fig. 2-7, lanes 2 and 3). The abundance of the amplification products was dependent upon the concentration of the template CMS-Sprite mtRNA. As the concentration of the template mtRNA decreased to 1.5 μg

per reaction, RT-PCR product was greatly reduced (see Fig. 2- 10, lane 2 and lane 3). The possibility that the RT-PCR products might be amplified from contaminating DNA was ruled out by negative control (Fig. 2-8, lane 1) in the absence of RT. Therefore the sublimon mtDNA was transcribed into RNA. We can conjecture that the 18S/5S secondary structure may hinder reverse transcriptase from extending efficiently into the 18S/5S rRNA located at 5' of the *pvs*.

To ask if the sublimon molecule containing the 18S/5S rRNA genes and the *pvs* was responsible for the CMS-unique 7.0 kb transcript, northern hybridization analysis was performed with mtRNA of CMS-Sprite, Mo-Triumph, and WPR-1 with DNA probes covering the18S/5S rRNA genes. Probing with the 18S/5S rRNA-specific probes did not reveal the 7.0 kb transcript. End-labeled oligonucleotides and a 5S-specific DNA probe of 112 bp were used for northern hybridization to eliminate the possibility of the intra-molecular secondary structure of the probes. However, these probes were not able to confer enough intensity to detect the 7.0 kb *pvs* transcript. This was perhaps because the abundance of the *pvs* transcript was relatively very low compared to that of the 18S/5S rRNA. Whether the sublimon molecule containing the 18S/5S rrn and *pvs* is responsible for the CMS-unique 7.0 kb transcript is still to be determined.

Discussion

The 2.1 kb Sequence of Unknown Origin Was Located at the 5' end of the 7.0 kb *pvs* Transcript.

In this study, we have investigated the origin of the 7.0 kb *pvs* transcript, which is CMS-unique and the most abundant *pvs* transcript in *P. vulgaris*. Immunohistochemical studies demonstrated that the ORF239 protein from the *pvs* transcript is expressed only in reproductive tissues of CMS lines (Abad et al., 1995). Some transgenic tobacco plants expressing *orf239* gene constructs are male sterile and show cell wall depositions of ORF239 protein. Even though previous studies (He et al., 1996; Abad et al., 1995) demonstrated that ORF239 protein from the *pvs* transcript is responsible for CMS, identification of the origin of the 7.0 kb *pvs* transcript is still important to understand expression of the *pvs* region. At least a 2.1 kb segment of a CMS-unique 7.0 kb transcript is not accounted for by northern hybridization with *pvs* or flanking DNA clones. The unidentified 2.1 kb of the 7.0 transcript could generate CMS-unique novel reading frames in addition to those identified. The location of this 2.1 kb at the 5' end of the *pvs* transcript means the promoter of the transcript has yet to be identified. Therefore, characterization of the 2.1 kb segment at the nucleotide sequence level is key to understand the molecular basis of CMS system in *P. vulgaris*.

To determine the origin of the unidentified 2.1 kb segment on the 7.0 kb transcript, oligonucleotide-directed RNase H digestion assays were performed with mtRNA of CMS-Sprite. The oligo-directed RNase-H cleavage experiments demonstrated that the 7.0 kb *pvs* transcript was linear and that the 2.1 kb segment in question was located in the region 5' of the *pvs* sequence. These two observations suggest two possibilities for the location of the 2.1 kb segment to the 5'of the *pvs*. One is that the unidentified 2.1 kb segment could be spliced 5' to the *pvs* sequence. This is plausible since trans-splicing processes have been observed in the generation of mature NADH dehydrogenase (*nad*) subunits 1, 2, and 5 transcripts in plant mitochondria (Chapdelain and Bonen, 1991; Wissinger et al., 1991; Knoop et al., 1991). Another possible explanation was that a mtDNA configuration such as a 'sublimon molecule', which was not observed in previous southern analysis, may exist and give rise to the 7.0 kb *pvs* transcripts. Due to recombination at repeated sequences, the plant mitochondrial genome could exist in many different configurations, albeit at varying stoichiometry (Small et al., 1989). Variation in the relative stoichiometry of mtDNA has been reported and shown to be widespread in plant cytoplasms (Morgens et al., 1984; Small et al., 1987). More than five repeat sequences active in recombination have been identified in maize

cytoplasms including Texas (T) cytoplasm, giving rise to various isomeric forms and subgenomic DNA molecules probably with different stoichiometry (Fauron et al., 1990b; Lonsdale et al., 1984).

Search for the Unidentified 2.1 kb Segment by 5'RACE

5'- rapid amplification of cDNA ends (RACE) initiated from primers within the *pvs* region was used to investigate the identity of the 2.1 kb segment located at the 5' end of *pvs* transcripts. Three cDNA clones carrying 26S rRNA sequences were obtained from 5' RACE cloning. As Northern blot hybridization with the 26S rRNA sequence as a probe could not verify that the identified 26S rRNA sequence was part of the 7.0 kb *pvs* transcript, we suspected that these RACE clones resulted from artifacts due to errors in the reverse transcription. Failure of cDNA synthesis from the 7.0 kb *pvs* transcript could be due to strong secondary structure suggested by primer extension analyses. High G-C contents were found in the mitochondrial DNA 5' to the *pvs* region. The recovery of bona fide 5'RACE clones from the 6.8 kb, CMS-unique transcript suggests secondary structures unique to the 7.0 kb *pvs* RNA. This RNA secondary structure possibly could prevent reverse transcriptase from extending into the unique 5' region of this transcript.

Transcription Mapping of the CMS-Sprite Mitochondrial Genome

An alternative approach to identify the 2.1 kb segment of the 7.0 kb *pvs* transcript, was carried out. This alternative approach was transcript mapping using WPR-3 cosmids as probes. Overlapping cosmid clones covering the entire mitochondrial genome of the male-fertile revertant line (WPR-3) were hybridized as DNA probes to Northern blots of CMS-Sprite and WPR1 mtRNA. The CMS-unique, 7.0 kb transcript in CMS-Sprite RNA was not detected by any of the WPR-3 cosmids. There is a possibility that a second, unidentified CMS-unique sequence could be present in the CMS-Sprite. As the WPR-3 cosmids did not detect the CMS-unique 7.0 kb transcript, the unidentified 2.1 kb segment was not represented on these cosmids.

Probing with cosmid clones revealed coding capacity and transcript patterns of the mitochondrial genome. Both simple and complex transcript patterns were observed in the mitochondria of *P. vulgaris*. Plant mitochondrial genes often show complex transcription patterns (reviewed by Gray et al., 1992). Makaroff and Palmer (1987) reported a simple transcription pattern of the *Brassica campestris* (218 kb) mitochondrial genome. They have showed that 24 abundant and non-overlapping transcripts account for approximately 30% of the mitochondrial genome. In

the larger *P. vulgaris* mitochondrial genome, 36 major abundant transcripts were revealed to cover 23% (97 kb) of the mitochondrial genome. Even though the size of *P. vulgaris* mitochondrial genome (approximately 417 kb) is twice as much as that of *B. campestris* (218 kb) mitochondrial genome, their coding capacity did not seem to increase. This suggested that coding capacity of plant mitochondria may be constant regardless of their genome size. As described above, DNA recombination and gene import may contribute to mitochondrial genome expansion (Fauron et al., 1995a; Palmer and Hebron, 1986; Stern and Palmer, 1984; Schuster and Brennicke, 1994). The function of many transcripts is still unknown, and might contain a number of as-yet-unidentified mitochondrial genes as suggested by the numerous open reading frames in the Arabidopsis mitochondrial genome (Unseld et al., 1997). Transcript maps would be alternative tools to localize plant mitochondrial genes on a complex map.

The dispersed distribution of transcripts in the 417 kb mitochondria genome in *P. vulgaris* suggested a large number of promoters. For instance, 18 transcription initiation sites were estimated in *O. berteriana* by capping assays (Binder and Brennicke, 1993), far exceeding the number of initiation sites in fungal and animal mitochondria. The majority of about 100 plant mitochondrial genes are probably transcribed as polycistronic RNAs

(Makaroff and Palmer, 1987). The large number of transcripts could be due, in part, to repeated sequences shared among transcripts, for example *atp9* gene sequences also represented in the chimeric, CMS-associated *pcf* gene in petunia (Young and Hanson, 1991), and may also result from transcription of multiple gene copies (four copies of *atp9* gene in this study). The maize *cob* and *atp9* genes have multiple transcription initiation sites (Mulligan et al., 1988). However, rRNA genes (26S and 18-5S) have a single major initiation site. A large number of mature rRNAs are processed from single pre-rRNA (Mulligan et al., 1988). Given the complexity of the plant mitochondrial genome, it is not easy to see how plant mitochondria maintain genome organization and transcribe consistent, but complex RNAs.

Sublimons Containing the *pvs* Sequence and 18S/5S rRNA Genes

A second possible explanation for the origin of the 7.0 kb CMS transcript was that a mtDNA configuration, which was not observed in previous southern analysis, may exist and give rise to this transcript. We investigated this possibility by southern blot hybridization and PCR analysis based upon a sublimon configuration in soybean mitochondria. The results presented here indicated that the mitochondrial *18S/5S* rRNA genes were joined to the 3' region of *orf209* and the downstream *pvs* sequence in

common bean (Fig. 2-9). The point of divergence between the o*rf209* and 5S rRNA gene was consistent with the very region where the 7.0 kb CMS transcript diverges from the CMS mtDNA configuration. A novel soybean mitochondrial transcript results from a mitochondrial DNA configuration containing 18S/5S rRNA genes and *orf214* (Morgens et al., 1984). In soybean, a restriction fragment containing the 18S/5S rRNA gene (2.0 kb) and the 3'region of *orf214* is present at low abundance in hypocotyls of 'Mandarin' cultivar, but is present as a predominant form in a tissue culture line of the same cultivar. The 3' region of *urf214* in soybean mitochondria could be a small recombinationally active repeat. This predominant form in the tissue culture line could be selectively amplified from the pre-existing sub-stoichiometric molecule containing 18S/5S and 3'region of *orf214* (Morgens et al., 1984). Therefore, the 18S/5S/*pvs* sublimon configuration detected in the CMS-Sprite of common bean mitochondria could also be selectively amplified in different environments like tissue culture and could confer a novel mitochondrial transcript as shown in soybean.

Mitochondrial genomes of soybean and common bean are similar in term of the *atpA/orf209* configuration. The entire *orf214* was also identified immediately 3' to the abundant soybean mitochondrial *atpA* gene (Chanut et al., 1992). The *orf209* homologous frames in both species are overlapped

with the terminal nucleotide of the *atpA* reading frame. Common-bean *orf209* shared 79% nucleotide sequence homology with the soybean *orf214* (Chase and Ortega, 1992). Two species are closely related in terms of mtDNA evolution as shown in the study of *coxII* gene transfer to the nucleus (Nugent et al., 1991).

The molecular heterogeneity of the common-bean mitochondrial genome could be generated by homologous recombination at repeated regions in the mtDNA. The possibility that the sublimon molecule arose from the mitochondrial genome via homologous recombination is suggested by the presence of the small repeats (3' *orf209 of* 282 bp) as well as large repeats (*atpA/orf209* and upstream region; 3'cob and downstream region) in two different configurations of the soy bean and common bean mitochondrial genomes. The 'sublimon' molecule could be generated by infrequent recombination events between short repeats (the 282 bp region of the 3' *orf209* in this study).

Whether repeat x and repeat y are recombinationally active or is still uncertain. However, we could surmise that two repeats could be used for mitochondrial recombination resulting in the loss of the *config-pvs* in revertant WPR-1 line. The three-stage recombination model involving

substoichiometric intermediates (Small et al., 1989) can be applied to the deletion of the *pvs* configuration in common bean mitochondrial genome.

To ask if the sublimon molecule containing the 18S/5S rRNA genes and the *pvs* is responsible for the CMS-unique 7.0 kb transcript, northern hybridization analysis was performed with mtRNA of CMS-Sprite and Mo-Triumph, WPR-1 with DNA probes covering the18S/5S rRNA genes. Northern analysis did not showed that the 7.0 kb transcript is transcribed from the sublimon mitochondrial DNA configuration. However, RT-PCR analysis showed that the sublimon mtDNA was transcribed into RNA. Thus, we can conjecture that the 18S/5S secondary structure may hinder the 18S/5S rRNA probes from hybridizing to the *pvs* for the northern blot. This could explain why 5-RACE technique could not produce clones representing the 18S/5S and *pvs* sublimon. Another consideration is the high abundance of 18S/5S transcripts relative to the 7.0 kb *pvs* transcript.

An alternative origin of the 7.0 kb *pvs* transcript might be transcripts that are imported from nuclear RNA and trans-spliced to the mitochondrial *pvs* region. Further investigation into the origin of the 7.0 kb *pvs* transcript will be required for characterization of CMS system in *P. vulgaris*.

Table 2-2. Oligonucleotides

Primer	Sequence	Location	Purpose
cc99	5'-GAGCTTTCTCAATGGCCCTTT	1076 [a]	*Orf214* region
cc100	5'-ATGCCGCGGTGAATATGTACC	517 [a]	PCR of 18S rRNA region
cc101	5'-ACACCCGATCCCATTCCGACC	931 [a]	PCR of 5S rRNA region
cc102	5'-GGTCGGAATGGGATCGGGTGT	951 [a]	PCR of 5S rRNA region
cc103	5'-GGGAGTACGGTCGCAAGACCG	880 [b]	PCR of 18S rRNA region
cc104	5'-AAGACAAACCGGGCACTACGG	897 [a]	PCR of 5S rRNA region
cc105	5'-GCTTTTACCATGTCTCCCGAACAACAATTTCAG	1012 [a]	PCR of 5S rRNA region
cc106	5'-TGTGTACTGTTCTTTTCGAA	-192 [c]	PCR of 18S rRNA region
cc107	5'-CTAAGCATCATTGGCTTGGTC	322 [b]	PCR of 18S rRNA region
cc108	5'-GAGTTTGATCCTGGCTCAGAA	17 [b]	PCR of 18S rRNA region
oligo2 (cc13)	5'-CTCCCTTCA AGGAAG	2163 [d]	RNase H digestion assay
oligo3 (cc11)	5'-TGCCTAAAGGACCCC	2903 [d]	RNase H digestion assay
oligo4 (cc43)	5'-GCTAGTCCTATTGAG	4502 [d]	RNase H digestion assay
neb1211	5'-CGGAAGATTGAGTTCCTG	Universal	T3 primer for sequencing
cc33	5'-CAGGAAACAGCTATGACC	Universal	T7 primer for sequencing
cc55	5'-CCATCTCGAAAGCACTAGGATCCG	2349 [d]	*pvs* primer for PCR & sequencing
cc68	5'-CCACTGCCTACATAACTAGACTCCC	2283 [d]	*pvs* primer for PCR & sequencing

[a] : Morgens et al., 1994
[b] : Grabau, 1985
[c] : Giese et al., 1996
[d] : Chase and Ortega, 1992

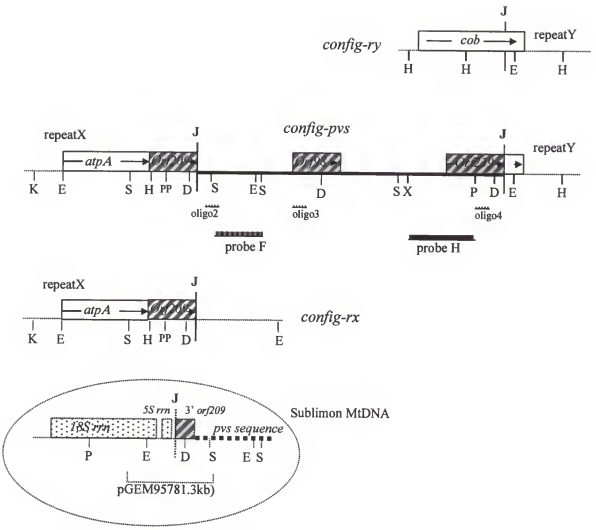


Fig. 2-1. Mitochondrial DNA configuration containing the *pvs* sequence and flanking repeats. Relative positions of oligos (.....) are shown in the *pvs* sequence (*solid line*). Junctions designated J represent divergent points between the repeated and *pvs* sequence on each configuration. Restriction recognition sites are designated as: *Dra*I (D), *Eco*RI (E), *Hind*III (H), *Kpn*I (K), *Pst*I (P), *Xho*I (X), and *Sst*I (S). The diagonally striped boxes represent *orfs* of unknown origin. The open boxes represent coding regions of essential mitochondrial genes. *Arrows* indicate the 5' to 3' orientations of *orfs* and genes. Dashed boxes placed below the restriction maps indicate DNA probes F and H. The configuration within the *dotted circle* indicates a putative sublimon molecule containing the *pvs* and 18S/5S rRNA coding sequence discussed below.

Fig. 2-2. Diagrammatic representation of the oligonucleotide-directed RNase H cleavage assay. RNAs were annealed with specific oligonucleotides (▁▁) and digested with RNase H. RNase H digests RNA of RNA/DNA hybrids, resulting in a nick of the RNA molecule. The nicked RNA fragments were denatured with glyoxal, separated by agarose gel electrophoresis, immobilized to a membrane, and hybridized with radio-labeled DNA probes (▬)spanning the nicked regions. If the RNA exists as a linear molecule, then this treatment results in two bands on the autoradiograph, and bands differ in size depending on the location of the oligos. If the transcript is a circular molecule, RNase H digestion would yield only one band, which is the same in size despite different oligos used. If the RNA is an aberrant or lariat form, migration patterns of the nicked products would not be consistent with that of intact RNA (Capel et al, 1993; Backert et al., 1996).

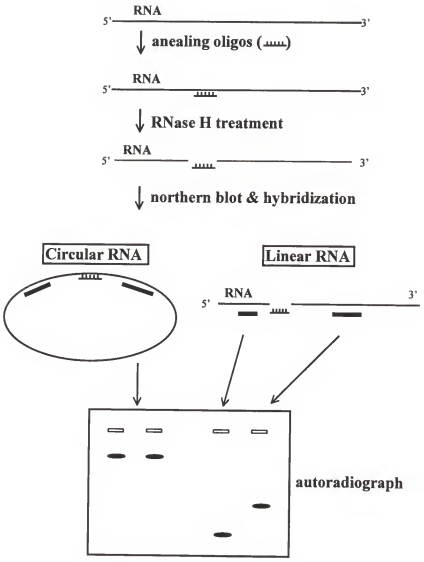
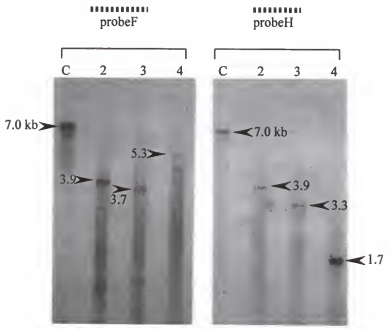
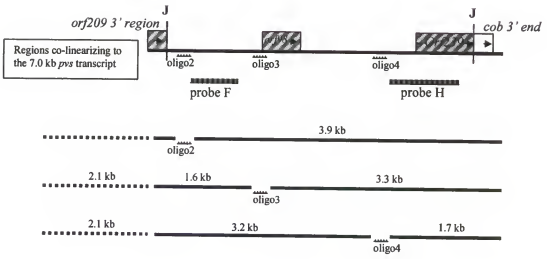

RNA
5'
3'
anealing oligos (
RNA
5'
3'
RNase H treatment
RNA
5'
3'
northern blot & hybridization
Circular RNA
Linear RNA
RNA
5'
3'
autoradiograph

Fig. 2-3. Oligonucleotide-directed RNase H digestion assay. (A) The northern blots from oligonucleotide-directed RNase H digestion. (B) Diagrammatic representation of the assays and the relative position of the unidentified 2.1 kb segment of the 7.0 kb *pvs* transcript. Physical maps of the mitochondrial DNA of CMS-Sprite and relative position of oligos annealed to the *pvs* region are shown in Fig. 1. Total mitochondrial RNA from CMS-Sprite was annealed with oligos 2, 3, and 4, treated with RNase H as described by Capel et al (1993), denatured with glyoxal, separated by agarose-gel electrophoresis, and blotted to nylon supports. Each ^{32}P-labeled DNA probe F and H (dashed boxes) was hybridized separately after one another to the same RNA blot (panel A). Lane C contains the uncut RNAs of CMS-Sprite for control. Other lanes contain RNAs that have been digested by RNase H at the positions of annealed oligo 2 (lane 2), oligo 3 (lane 3), oligo 4 (lane 4). Relative positions of oligos (▬) are shown in the *pvs* sequence. The fragments generated by oligo-annealed digestion are shown below the maps, along with the putative junction between the *pvs* region and unidentified 2.1 kb. The diagonally striped boxes represent *orfs* of unknown origin. The open boxes represent portions of the coding regions. Arrows indicate the 5' to 3' orientations of *orfs* and genes.

A


probeF
probeH
C 2 3 4
C 2 3 4
7.0 kb
5.3
3.9
3.7
7.0 kb
3.9
3.3
1.7

B


orf209 3' region
J
J
cob 3' end
Regions co-linearizing to the 7.0 kb pvs transcript
oligo2
oligo3
oligo4
probe F
probe H
3.9 kb
oligo2
2.1 kb
1.6 kb
3.3 kb
oligo3
2.1 kb
3.2 kb
1.7 kb
oligo4

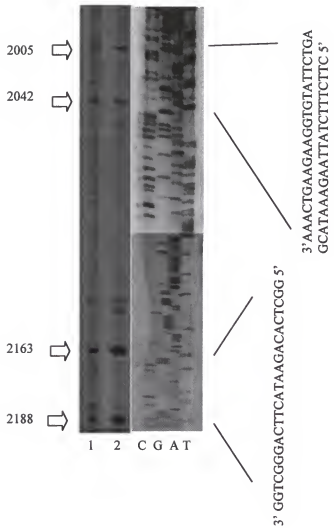
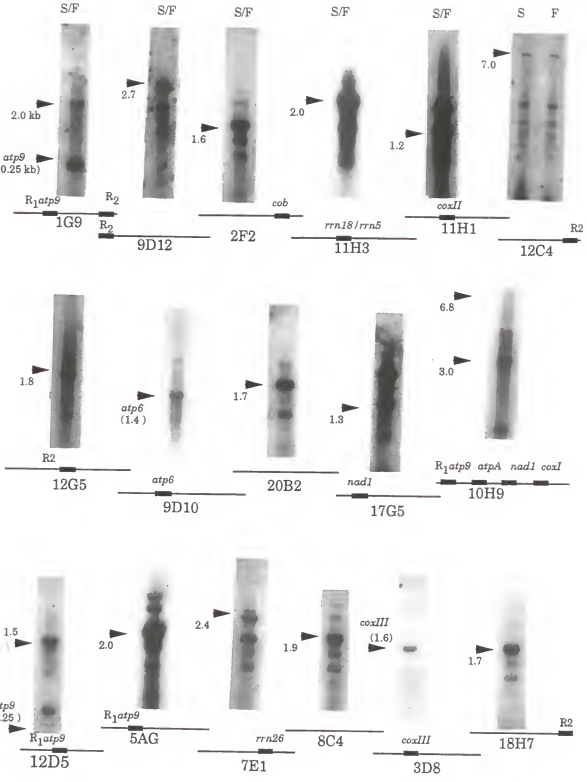


Fig. 2-4. Primer extension analysis of CMS-Sprite RNA with oligo 2 (Fig. 2-1). The extension reaction was conducted at 42 °C in the presence (lane 1) or absence (lane 2) of the denaturants (methyl-mercury). *Numbers* on the left indicate positions of the bands started from first nucleotide of initiation codon in *atpA* coding sequence. The extended products were electrophoresed with the sequence ladder extended from the same primer annealed to *pvs* DNA clone.

Fig. 2-5. Hybridization of mitochondrial RNAs with WPR-3 cosmid library clones. Seedling mitochondrial RNAs from CMS-Sprite (S) and male-fertile WPR-1 (F) were denatured with glyoxal, separated by agarose gel electrophoresis, blotted to nylon supports and hybridized with radio-labeled probes. Individual cosmid clones were digested with *Pst*I, phenol-extracted and alcohol-precipitated. The resulting fragments were labeled with ^{32}P-dCTP and hybridized to the RNA blots. Intensities of hybridizing bands are not comparable between blots. The clone numbers and placement of genes is according to Janska and Mackenzie (1993).


S/F
S/F
S/F
S/F
S/F
S
F
2.0 kb
atp9
(0.25 kb)
2.7
1.6
2.0
1.2
7.0
R1atp9
R2
cob
coxII
1G9
R2
9D12
2F2
rrn18/rrn5
11H3
11H1
R2
12C4
1.8
R2
12G5
atp6
(1.4)
atp6
9D10
1.7
20B2
1.3
nad1
17G5
6.8
3.0
R1atp9 atpA nad1 coxI
10H9
1.5
atp9
(0.25)
R1atp9
12D5
2.0
R1atp9
5AG
2.4
rrn26
7E1
1.9
coxIII
(1.6)
8C4
coxIII
3D8
1.7
R2
18H7

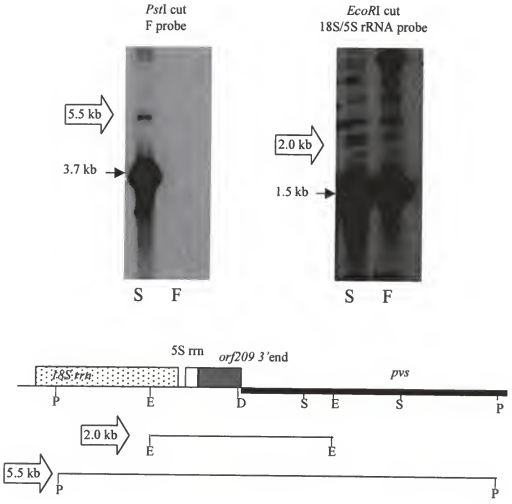


Fig. 2-6. Identification of sub-stoichiometric fragments containing the *pvs* sequence in common bean mtDNA. MtDNAs from CMS-Sprite (lane S) and revertant WPR-1 (lane F) were digested with *PstI* and *EcoRI*, separated by agarose gel electrophoresis, stained with ethidium bromide, blotted to membrane supports and hybridized with a radio-labeled probes of the *pvs* and *18S/5S* rRNA sequence, respectively. *Open arrows* on the left indicate CMS-unique sub-stoichiometric fragments in CMS-Sprite. Lower panel indicates schematic representation of the predicted sublimon configuration containing the 18S/5S rRNA genes and the *pvs* sequence.

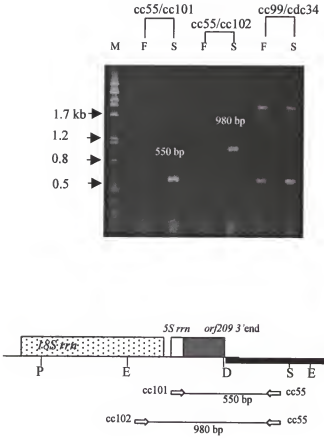


Fig 2-7. PCR amplification from mtDNA containing 18S/5S genes and the *pvs* sequence. MtDNAs from CMS-Sprite (S) and fertile WPR-1 (F) were used for the PCR reaction with different sets of primers. Primer pair cc99/cdc34 was used for positive control to amplify the major *atpA/orf209* region that exists in both CMS-Sprite and WPR-1. Sequence analysis verified that two PCR products result from the inverted repeats of the cc99 primer sequence in 5' *atpA* and 3' *orf209*. PCR products were electrophoretically separated in a 0.8 % agarose gel and are shown as ethidium bromide staining patterns. Molecular-weight marker (M) was λ(lamda) DNA cut by *Pst*I. Lower panel shows a schematic view for the PCR products obtained with primer pairs cc55/cc101 or cc55/cc102 (see Table 1).

Fig. 2-8. Alignment of the novel sublimon mtDNA containing the 18S/5S rRNA genes, 3' *orf209* and the *pvs* sequence in *P. vulgaris*, with corresponding soybean pSB2-2 mitochondrial DNA sequences. Numbers to the left refer to nucleotides in the soybean mitochondrial sequence (Morgens et al., 1984) or the common bean mitochondrial sequence (Chase and Ortega, 1992). This configuration in soybean is predominant during growth in tissue culture, while it is of low abundance in mtDNA of 'Mandarin' hypocotyls. *Horizontal lines* above the nucleotide sequence indicate coding regions. The *dots* indicate the absence of a corresponding nucleotide in the sequence. The *large letters* indicate the mismatching nucleotides in 18S/5S rRNA genes between two species. *Italicized letters* indicate part of *orf209/orf214* sequence. ✯ Indicates an in-frame termination codon in *orf209* and *orf214*. Junction designated J represents divergence point between 18S/5S rRNA genes and the *orf209/pvs* in common bean.

```
                TGATCACCCA TGACTTCTGT GTACCACTAG TGCCACAAAG GCTTTTGGTG      Common Bean
601             TGATCACCCA TGACTTCTGT GTACCACTAG TGCCACAAAG GCTTTTGGTG      SoyBean

                GTCTTATTGG CGCATACCAC GGTGGGGTCT TCGACTGGGG TGAAGTCGTA      CB
651             GTCTTATTGG CGCATACCAC GGTGGGGTCT TCGACTGGGG TGAAGTCGTA      SB

                                    18S rRNA gene
                ACAAGGTAGC CGTAGGGGAA CCTGTGGCTG GATGGAATCC TT|CGCGATGG     CB
701             ACAAGGTAGC CGTAGGGGAA CCTGTGGCTG GATGGAATCC TT|CGCGATGG     SB

                AAAAGCCCTC TTCCCCCAAC GGGGCTAGCT TGCTGGAATG TGGGCTTTGG      CB
751             AAAAGCCCTC TTCCCCCAAC GGGGCTAGCT TGCTGGAATG CGGGCTTTGG      SB

                GTCCAAAGGA CACCGGGGGC GAGTCAAAAA AATCGTTCCA TTTTCCCTTC      CB
801             GTCCAAAGGA CACCGGGGGC GAGTCAAAAA AATCGTTCCA TTTTCCCTTC      SB

                cCTTGGTCGAGCA CTTCGTTCCT TCTGGTTCAT CAATCGGAAA TAAATAAAAGA  CB
851             C...GGTCGAGCA CTTCGTTCCT TCTTGTTCAT CAATCGGAAA TCAAGCAAGA   SB

                cAAACCGGGC ACTACGGTGA GACGTGAAAA CACCCGATCC CATTCCGACC      CB
901             cAAACCGGGC ACTACGGTGA GACGTGAAAA CACCCGATCC CATTCCGACC      SB

                TCGATATGTG GAATCGTCTT GCGCCATATG TACTGAAATT GTTCGGGAGA      CB
951             TCGATATGTG GAATCGTCTT GCGCCATATG TACTGAAATT GTTCGGGAGA      SB

                                        J  position # 1851
                5S rRNA gene
                CATGGTAAAAGC TCGGAA GA GAAGAAAGAAAAAGTGCTTT TGGCACAAGT      CB
1001            CATGGTC AAAGC TCGGAA G .....AAAAGTGCTAA ACGCACAAGT          SB
                                      orf209/orf214

1883            CCAAATAGAA AGGGCCATTG AGAAAGCTCT GCTCTCCGAC GGGTATTCCC      CB
1041            CAAAATCGAA AGGGCCATTG AGAAAGCTCT GCTCTCCGAC GGGTATTCCC      SB

1933            GGGATGAGCT TTCTCAAAGG AGCAAACGAG ATGAGATTAG GGGCTTTTTG      CB
1091            GGGATGAGCT TTCTCAAAGG AGCAAACGAG ATGAAAGAAG GACCTTGTTG      SB

1983            TTCTACCGTA ATGGGAAACT TCTTTCTATT AAGAAATACG AGTCTTATGT      CB
1141            TTCTACCGTA CTGGGAAACT TCTTTCGATG AAAACATATG ACTCATATGT      SB

2033            GGAAGAAGTC AAATTTGGGA CCCACCGCAG CCAGCCATAT CAAGATCTTA      CB
1191            AAAAGAAGTC GAATTAGGGA CCCACCGGAG CCAACCCTAT AAGGTTCTTA      SB

2083            TCCATGCCAT CTCTGATTCC TATCTTTTTT TAAAGAAAGT AAAAAAAATA      CB
1241            TCAATGCCAT CTCTTCTTCC AATCTTTTTT TAAAGAAAGT CAAAAAAATC      SB

                                    ☆                  pvs sequence
2133            AAGAGGTGGGAACGGGGGTGAAACAG|ATTGGGCTCACAGAATACTTCAGGG       CB
1291            AAGAGGTGGGAATTAGGAAG..ACAG TGGGAACAGTGGGGGG.GGAGAAGGTGA    SB
                                                                    ☆
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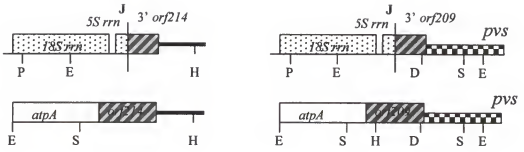

Tissue culture line SB-1 of soybean

Common bean

Fig. 2-9. Comparison of the mtDNA organizations containing the *atpA/orf209* sequence and *18S/5S/3'orf209* between common bean and soybean.

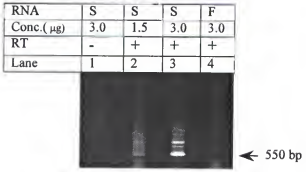

Fig. 2-10. RT-PCR analysis revealed the presence of RNA transcribed from the 18S/5S rRNA and *pvs* configuration. Mitochondrial RNAs from CMS-Sprite (S) and male-fertile WPR-1 (F) were used for the RT-PCR reaction in the presence (+) or absence (-) of reverse transcriptase (RT). Lane 1, the CMS-Sprite RNA (3.0 μg) without RT for negative control; lane 2, CMS-Sprite RNA (1.5 μg); lane 3, CMS-Sprite (3.0 μg); lane 4, WPR-1 RNA (3.0 μg). RT-PCR products resulting from the use of the cc55 and cc101 primers (Fig. 2-7) were electrophoretically separated in a 0.8 % agarose gel and are shown as ethidium bromide staining patterns.

CONCLUSION AND FUTURE PROSPECTIVES

This study addressed two major objectives on the CMS-unique *pvs* transcripts to understand CMS systems in higher plants. I identified the CMS-unique transcripts that contain the *atpA*/*orf209* coding sequence and the *pvs* sequence. I have shown that three C-to-U partial RNA editing event in the *orf239* coding region and three complete editing sites of the 3' *cob* segment occurred in the *pvs* transcripts. The concomitant occurrence of partial and complete editing patterns found in the *pvs* transcripts suggested that transcript abundance or stability does not affect the frequency of RNA editing for these transcripts. RNA editing in *orf239* and presence of the *orf239* homologous sequences in the mitochondria of other plant species raise the possibility that the *orf239* could be part of as-yet-unidentified mitochondrial protein coding gene in plants. In conclusion, RNA editing did not alter or create the novel open reading frame in the *pvs* transcript.

I also identified a 'sublimon' DNA configuration in which the mitochondrial 18S/5S rRNA sequence was fused to the 3' portion of the *orf209* and the *pvs* sequence. RT-PCR verified the transcription of this

configuration in CMS-Sprite and G08063, but not in WPR-1. This could be the first evidence that sublimon mtDNA can be transcribed. Since the 18S/5S rRNA promoter is one of the strongest in plant mitochondria, it could give an abundant transcript even from the sublimon molecule. Further study would be required to show that the *pvs* 7.0 kb resulted from the CMS-unique sublimon mtDNA configuration with the 5S rRNA gene and the *pvs* sequence.

REFERENCES

Abad, A. R., Mehrtens, B. J., and Mackenzie, S. A. (1995). Specific expression in reproductive tissues and fate of a mitochondrial sterility-associated protein in cytoplasmic male-sterile bean. Plant Cell 7, 271-285.

Akagi, H., Sakamoto, M., Shinjyo, C., Shimada, H., and Fujimura, T. (1994). A unique sequence located downstream from the rice mitochondrial atp6 may cause male sterility. Curr. Genet. 25, 52-58.

Andre, C., Levy, A., and Walbot, V. (1992). Small repeated sequences and the structure of plant mitochondrial genomes. TIG 8, 128-132.

Attardi, G. (1988). Biogenesis of mitochondria. Ann. Rev. Cell Biol. 4, 289-333.

Backert, S., Dorfel, P., Lurz, R., and Borner, T. (1996). Rolling-circle replication of mitochondrial DNA in the higher plant *Chenopodium album* (L.). Mol. Cellular Biol. 16, 6285-6294.

Bailey-Serres, J., Hanson, D. K., Fox, T. D., and Leaver, C. J. (1986). Cell 47, 567-576.

Bassett, M. J., and Shuh, D. M. (1982). Cytoplasmic male sterility in common bean. J. Am. Soc. Hort. Sci. 107, 791-793.

Begu, D., Graves, P. V., Domec, C., Arselin, G., Litvak, S., and Araya, A.(1990). RNA editing of wheat mitochondrial ATP synthase subunit 9: direct protein and cDNA sequencing. Plant Cell, 1283-1290.

Belliard, G., Pelletier, G., Vedel, F., and Quetier, F., (1978). Morphological characteristics and chloroplast DNA distribution in different cytoplasmic parasexual hybrids of *Nicotiana tabacum.* Mol. Gen. Genet. 165, 231-37.

Bendich, A. (1993). Reaching for the ring: the study of mitochondrial genome structure. Curr. Genet. 24, 279-290.

Bendich, A. (1996). Structural analysis of mitochondrial DNA molecules from fungi and plants using moving pictures and pulsed-field gel electrophoresis. J. Mol. Biol. 255, 564-588.

Bendich, A., and Smith, S. B. (1990). Moving pictures and pulsed-field gel electrophoresis show linear DNA molecules from chloroplasts and mitochondria. Curr. Genet. 17, 421-425.

Benne, R., Van Den Burg, J, Brakenhoff, J. P. J., Sloof, P., Van Boo, J. H., and Tromp, M. C. (1986). Major transcript of the frameshifted *coxII* gene from trypanosome mitochondria contains four nucleotides that are not encoded in the DNA. Cell 46, 819-826.

Binder, S., and Brennicke, A. (1993). Transcription initiation sites in mitochondria of *Oenothera berteriana.* J. Biol. Chem. 268, 7849-55.

Binder, S., Hatzack, F., and Brennicke, A. (1995). A novel pea mitochondrial *in vitro* transcription system recognizes homologous and heterologous mRNA and tRNA promoters. J. Biol. Chem. 270, 22182-89.

Boeshore, M. L., Liftshitz, I., Hanson, M. R., and Izhar, S. (1983). Novel composition of mitochondrial genomes of Petunia somatic hybrids derived from cytoplasmic male sterile and fertile plants. Mol. Gen. Genet. 190, 459-467.

Brown, G. G., Auchincloss, A. H., Covello, P. S., Gray, M. W., Menassa, R., and Singh, M. (1991). Characterization of transcription initiation sites on the soybean mitochondrial genome allows identification of a transcription-associated sequence motif. Mol. Gen. Genet. 228, 345-355.

Capel, B., Swain, A., Nicolis, S., Hacker, A., Walter, M., Koopman, P., Goodfellow, P., and Loveii-Badge, R. (1993). Circular transcript of the testis-determining gene *Sry* in adult mouse testis. Cell 73, 1019-1030.

Chanut, F. A., Grabau, E. A., and Gesteland, R. F. (1993). Complex organization of the soybean mitochondrial genome: recombination repeats and multiple transcripts at the *atpA* loci. Curr. Genet. 23, 234-247.

Chao, S., Sederoff, R. R., and LevingsIII, C. S. (1983). Partial sequence analysis of the 5S and 18S rRNA gene region of the maize mitochondrial genome. Plant Physiol. 71, 190-193.

Chapdelaine, Y., and Bonen, L. (1991). The wheat mitochondrial gene for subunit I of NADH dehydrogenase complex: a *trans*-splicing model for this gene-in-pieces. Cell 65, 465-472.

Chase, C. D. (1994). Expression of CMS-unique and flanking mitochondrial DNA sequences in *Phaseolus vulgaris* L. Curr. Genet. 25, 245-251.

Chase, C. D., and Ortega, V. M. (1992). Organization of *atpA* coding and 3' flanking sequences associated with cytoplasmic male sterility in Phaseolus vulgaris L. Curr. Genet. 2, 147-153.

Chaudhuri, S., Carrer, H., and Maliga, P. (1995). Site-specific factor involved in the editing of the psbmRNA in tobacco plastids. EMBO J. 14, 2951-2957.

Corens, C. (1909). Vererbungsversuche mit blass (gleb) rrunen und buntblattrigne sippen bei *Mirabilis jalapa, Urticua pilulifera und Lunaria auuua. Z. Vererbungs.* 1, 291-329. Cited by Gillham, N. W. (1978). In Organelle Heredity. Raven Press. New York. Cited by Chase, C. (1998) 'The discovery of plant mitochondrial genomes.' *in press.*

Covello, P. S., and Gray, M. W. (1989). RNA editing in plant mitochondria. Nature 341, 662-666.

Covello, P. S., and Gray, M. W. (1990). Difference in editing at homologous sites in mRNAs from angiosperm mitochondria. Nucleic. Acids Res. 18, 5189-5196.

Covello, P. S., and Gray, M. W. (1991). Sequence analysis of wheat mitochondrial transcripts capped *in vitro*: definitive identification of transcription initiation sites. Curr. Genet. 20, 245-251.

Covello, P. S., and Gray, M. W. (1992). Silent mitochondrial and active nuclear genes for subunit 2 of cytochrome c oxidase (*cox2*) in soybean: evidence for RNA-mediated gene transfer. EMBO J. 11, 3815-20.

Cui, X., Wise, R. P., and Schnable, P. S. (1996). The *rf2* nuclear restorer gene of male-sterile T-cytoplasm maize. Science 272, 1334-1336.

Dellaporta, S. L., Wood, J., and Hick, J. B. (1983). A plant DNA minipreparation: Version II. Plant Mol. Biol. Rep. 1, 19-21.

Dewey, R. E., Levings, C. S. III, and Timothy, D. H. (1986). Novel recombinations in maize mitochondrial genome produce a unique transcriptional unit in the Texas male-sterile cytoplasm. Cell 44, 439-449.

Dewey, R. E., and Levings, C. S. III. (1985). Nucleotide sequence of ATPase 6 gene of maize mitochondria. Plant Physiol. 79, 914-919.

Dewey, R. E., Timothy, D. H., and Levings, C. S. III. (1987). A mitochondrial protein associated with cytoplasmic male sterility in the T cytopalsm of maize. Proc. Natl. Acad. Sci. USA 84, 5374-5378.

Dombrowski, S., Brennicke, A., and Binder, S. (1997). 3'-inverted repeats in plant mitochondrial mRNAs are processing signals rather than transcription terminators. EMBO J. 16, 5069-5076.

Fauron, C. M. -R., Havlik, M., and Brettell, R. I. S. (1990a). The mitochondrial genome organization of a maize fertile cmsT revertant line is generated through recombination between two sets of repeats. Genetics 124, 423-428.

Fauron, C. M. -R., Havlik, M., and Brettell, R. I. S., and Albertsen, M. (1990b). Study of two different recombination events in maize events in maize cmsT-regenerated plants during reversion to fertility. Theor. Appl. Genet. 79, 593-599.

Fauron, C. M.-R., Casper, M., Gao, Y., and Moore, B. (1995a). The maize mitochondrial genome: dynamic, yet functional. Trends in Gent. 11, 228-235.

Fauron, C. M.-R., Moore, B., and Casper, M. (1995b). Maize as a model of higher plant mitochondrial genome plasticity. Plant Sci. 112, 11-32.

Feinberg, A. P. and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132, 6-13.

Folkerts, C. M. R., and Hanson, M. R. (1991).The male sterility-associated *pcf* gene and the normal *atp9* gene in *Petunia* are located on different sub-genomic mitochondrial DNA molecules. Genetics 129, 885-895.

Forsburg, S. L., and Guarente, L. (1989). Communication between mitochondria and the nucleus in regulation of cytochrome genes in the yeast *Saccharomyces cerevisie*. Annu. Rev. Cell Biol. 5, 153-180.

Fox, T. D. and Leaver, C. J. (1981). The *Zea mays* mitochondrial gene coding cytochrome oxidase subunit II has an intervening sequence and does not contain TGA codons. Cell 26, 315-323.

Giese, A., Thalheim, C., Brennicke, A., and Binder, S. (1996). Correlation of nonanucleotide motifs with transcript initiation of 18S rRNA genes in mitochondria of pea, potato, and *Arabidopsis*. Mol. Gen. Genet. 252, 429-436.

Gonzalez, D. H., Bonnard, G., and Grienenberger, J. M. (1993). A gene involved in the biogenesis of *c*-type cytochromes is co-transcribed with a ribosomal protein gene in wheat mitochondria. Curr. Genet. 21, 248-255.

Grabau, E. A. (1985). Nucleotide sequence of the soybean mitochondrial 18S rRNA gene: evidence for a slow rate of divergence in the plant mitochondrial genome. Plant Mol. Biol. 5, 119-124.

Gray, M. W., and Covello, P. S. (1993). RNA editing in plant mitochondria and chloroplasts. The FASEB J. 7, 64-72.

Gray, M. W., Pamela, J., Hanic-Joyce, and Covello, P. S. (1992). Transcription, processing and editing in plant mitochondria. Ann. Rev. Plant Physiol. Plant Mol. Biol. 43, 145-175.

Grohmann, L., Thieck, O., Herz, U., Schroder, W., and Brennicke, A. (1994). Translation of *nad9* mRNAs in mitochondria from *Solanum tuberosum* is restricted to completely edited transcripts. Nucleic Acids Res. 22, 3304-3311.

Grosskopf, D., and Mulligan, R. M. (1996). Developmental- and tissue-specificity of RNA editing in mitochondrial of suspension-cultured maize cells and seedlings. Curr. Genet. 29, 556-563.

Gualberto, J. M., Lamattina, L., Bonnard, G., Weil, J. -H., and Grienenberger, J. -M. (1989). RNA editing in wheat mitochondria results in the conservation of protein sequences. Nature 341, 660-662.

Gualberto, J. M., Weil, J. H., and Grienenberger, J. M. (1990). Editing of the wheat *coxII* transcript: evidence for twelve C to U and one U to C conversion nad sequence similarities around editing sites. Nucleic Acids Res. 18, 3771-3776.

Gualberto, J. M., Wintz, H., Weil, J. -H., and Grienenberger, J. -M. (1988). The genes coding for subunit 3 of NADH dehydrogenase and for ribosomal protein S12 are present in the wheat and maize mitochondrial genomes and are co-transcribed. Mol. Gen. Genet. 215, 118-127.

Hanson, M. R. (1991). Plant mitochondrial mutations and male sterility. Ann. Rev. Genet 25, 461-486.

Hanson, M. R., and Conde, M. F. (1985). Functioning and variation of cytoplasmic genomes: lessons from cytoplasmic-nuclear interaction affecting male fertility in plant. Int. Rev. Cytol. 94, 213-267.

Hanson, M. R., Sutton, C. A., and Lu, B. (1996). Plant organelle gene expression: altered by RNA editing. Trends in Plant Science 1, 57-64.

He, S., Abad, A. R., Gelvin, S. B., and Mackenzie, S. A. (1996). A cytoplasmic male sterility-associated mitochondrial protein causes pollen disruption in transgenic tobacco. Proc. Natl. Acad. Sci. USA 93, 11763-68.

Hernould, M., Suharsono, S., Litvak, S., Araya, A., and Mouras, A. (1993). Male-sterility induction in transgenic tobacco plant with an unedited *atp9* mitochondrial gene from wheat. Proc. Natl. Acad. Sci. USA 90, 2370-74.

Hervieu, F., Bannerot, H., and Pelletier, G. (1994). A unique male sterility (CMS) determinants is present in three *Phaseolus* species characterized by different mitochondrial genomes. Theor. Appl. Genet. 88, 314-320.

Hiesel, R., Wissenger, B., Schuster, W., and Brennick, A. (1989). RNA editing in plant mitochondria. Science 246, 1632-1634.

Hirose, T., and Sugiura, M. (1997). Both RNA editing and RNA cleavage are required for translation of tobacco chloroplast *ndhD* mRNA: a possible regulatory mechanism for the expression of a chloroplast operon consisting of functionally unrelated genes. The EMBO J. 16:6804-6811.

Hoch, B., Maier, R. M., Appel, K., Igloi, G. L., and Kossel, H. (1991). Editing of a chloroplast mRNA by creation of an initiation codon. Nature 353, 178-180.

Hodges, P.,and Scott, J. (1992). Apolipoprotein B mRNA editing: a new tier for the control of gene expression. Trends Biochem. Sci. 17, 77-81.

Howad, W., and Kempken, F. (1997). Cell type-specific loss of *atp6* RNA editing in cytoplasmic male sterile *Sorghum bicolor*. Proc. Natl. Acad. Sci. 94, 11090-11095.

Hunt, M. D., and Newton, K. J. (1991). The NCS3 mutation: genetic evidence for the expression of ribosomal protein genes in *Zea mays* mitochondria. EMBO J. 10, 1045-52.

Iwabuchi, M., Kyozuka, J., and Shimamoto, K. (1993). Processing followed by complate editing of an altered mitochondrial atp6 RNA restores fertility of cytoplasmic male sterile rice. EMBO J. 12, 1437-1446.

Janska, H., and Mackenzie, S. A. (1993). Unusual mitochondrial genome organization in cytoplasmic reversion to fertility. Genetics 135, 869-879.

Johns, C., Lu, M., Lyznik, A., and Mackenzie, S. (1992). A mitochondrial sequence is associated with abnormal pollen development in cytoplasmic male sterile bean plants. Plant Cell. 4, 435-449.

Joyce, P. B., and Gray, M. W. (1989). Chloroplast-like transfer RNA genes expressed in wheat mitochondria. Nucleic Acids Res. 17, 5461-5476.

Kanno, A., and Hirai, A. (1993). A transcription map of the chloroplast genome from rice (*Oryza sativa*). Curr. Genet. 23, 166-174.

Kempken, F., and Howad, W. (1996). Mitochondrial RNA editing is sequence specific and independent of transcript abundance in *Sorghum bicolor*. Curr. Genet. 30, 186-189.

Kempken, F., Hofken, G., and Pring, D. R. (1995). Analysis of silent editing sites in *atp6* transcripts of *Sorghum bicolor*. Curr. Genet. 27, 555-558.

Kempken, F., Mullen, J., Pring, D. R., and Tang, H. V. (1991). RNA editing of *Sorghum* mitochondrial *atp6* transcripts changes 15 amino acids and generates a carboxy-terminus identical to yeast. Curr. Genet. 20, 417-422.

Kennell, J. C., and Pring, D. R. (1989). Initaition and processing of *atp6, Turf-13* and *ORF25* transcripts from mitochondria of T-cytoplasm maize. Mol. Gen. Genet. 216, 16-24.

Knoop, CV., Schuste, W., Wissinger, B., and Brennicke, A. (1991). *Trans*-splicing integrates and exon of 22 nucleotides into the *nad5* mRNA in higher plant mitochondria. EMBO J. 10, 3483-3493.

Kubo, T., and Mikami, T. (1996). A duplicated sequence in sugarbeet mitochondrial transcripts is differentially edited: analysis of *orfB* and its derivative *orf324* mRNAs. Biochimica et Biophysica Acta 1307, 259-262.

Kumar, R., and Levings III, C. S. (1993). RNA editing of a chimeric mitochondrial gene transcript is sequence specific. Curr. Genet. 23, 154-159.

Lamattina, L., and Grienenberger, J. M. (1991). RNA editing of the transcript coding for subunit 4 of NADH dehydrogenase in wheat mitochondria: uneven distribution of the editing sites among the four exons. Nucleic acids Res. 19, 3275-3282.

Laser, K. D., and Lerstern, N. R. (1972). Anatomy and cytology of microsporogenesis in cytoplasmic male sterile angiosperms. Bot. Rev. 38, 425-454.

Levings, C. S. III (1993). Thoughts on cytoplasmic male sterility in cms-T maize. The Plant Cell 5, 1285-90.

Levings, C. S. III (1996). Infertility treatment: a nuclear restorer gene in maize. Science 272, 1279-1280.

Levy, A. A., Andre, C. P., and Walbot, V. (1991). Analysis of a 120-kilobase mitochondrial chromosome in maize. Genetics 128, 417-425.

Liao, X., and Butow, R. A. (1993). RTG1 and RTG2: Two yeast genes required for a novel path of communication from mitochondria to the nucleus. Cell 72, 61-71.

Lonsdale, D. M., Hodge, T. P., Fauron, and C. M. -R. (1984). The physical map and organization of the mitochondrial genome from the fertile cytoplasm of maize. Nucleic Acids Res. 12, 9249-61.

Lonsdale, D. M. (1989). The plant mitochondrial genome. In: The Biochemistry of Plant, vol15, p229-295, Academic Press, New York.

Lu, B., and Hanson, M. R. (1992). A single nuclear gene specifies the abundance and extent of RNA editing of a plant mitochondrial transcript. Nucleic Acids Res. 20, 5699-5703.

Lu, B., and Hanson, M. R. (1994). A single homogeneous form of ATP6 protein accumulation in petunia mitochondria despite the presence of differentially edited *atp6* transcripts. Plant Cell 6, 1955-1968.

Mackenzie, S. A. (1991). Identification of a sterility-inducing cytoplasm in a fertile accession line of *Phaseolus vulgaris* L. Genetics 127, 411-416.

Mackenzie, S. A., and Bassett, M. J. (1987). Genetics of fertility restoration in cytoplasmic male sterile *Phaseolus vulgaris* L. Theor. Appl. Genet. 74, 642-645.

Mackenzie, S. A., and Chase, C. D. (1990). Fertility restoration is associated with loss of a portion of the mitochondrial genome in cytoplasmic male-sterile common bean. Plant Cell 2, 905-912.

Mackenzie, S. A., Pring, D. R., Bassett, M. J., and Chase, C. D. (1988). Mitochondrial DNA rearrangement associated with fertility restoration and cytoplasmic reversion to fertility in CMS *Phaseolus vulgaris* L. Proc. Natl. Acad. Sci. USA 85, 2714-2717.

Maier, R. M., Zeltz, P., Kossel, H., Bonnard, G., Gualberto, J. M., and Grienenberger, J. M. (1996). RNA editing in plant mitochondria and chloroplasts. Plant Mol. Biol. 32, 343-365.

Makaroff, C. A., and Palmer, J. D. (1987). Extensive mitochondrial specific transcription of the *Brassica campestris* mitochondrial genome. Nucleic Acids Res. 15, 5141-5156.

Makaroff, C. A., Apel, I. J., and Palmer, J. D. (1990). Characterization of radish mitochondrial *atpA:* influence of nuclear background on transcription of *atpA*-associated sequences and relationship with male sterility. Plant Mol. Biol. 15, 735-746.

Maloney, A. P. and Walbot, V. (1990). Structure analysis of mature and dicistronic transcripts from the 18S and 5S ribosomal RNA genes of maize mitochondria. J. Mol. Biol. 213, 6330-649.

Marechal-Drouard, L., Weil, J. -H., and Guillemaut, P. (1988). Import of several tRNAs from the cytoplasm into the mitochondria in bean *Phaseolus vulgaris.* Nucleic Acids Res. 16, 4777-88.

Meves, F. (1904). Uber das varkommen von mitochondrien bezw. Chondromitten in pflanzenzellen. Ber. Stsch. Bot. Ges. 22, 284-302. Cited by Douce, R. (1985) in Plant Mitochondria. Academic Press, Orlando. Cited by Chase, C. (1998) 'The discovery of plant mitochondrial genomes.' *in press.*

Moneger, F., Smart, C. J., and Lever, C. J. (1994). Nuclear restoration of cytoplasmic male sterility in sunflower is associated with tissue-specific regulation of a novel mitochondrial gene. EMBO J. 13, 8-17.

Morgens, P. H., Grabau, E. A., and Gesteland, R. F. (1984). A novel soybean mitochondrial transcript resulting from a DNA rearrangement involving the 5S rRNA gene. Nucleic Acids Res. 12, 5665-5684.

Mulligan, R. M., Leon, P., and Walbot, V. (1991). Transcriptional and post-transcriptional regulation of maize mitochondrial gene expression. Mol. Cel. Biol. 11, 533-543.

Mulligan, R. M., Maloney, A. P., and Walbot, V. (1988). RNA processing and multiple transcription initiation sites result in transcript size heterogeneity in maize mitochondria. Mol. Gen. Genet. 211, 373-80.

Nakazono, M., tadani, H., Wakasugi, T., Tsutsumi, N., Sugiura, M., and Hirai, A. (1995). The *rps3-rpl16-nad3-rps12* gene cluster in rice mitochondrial DNA is transcribed from alternative promoters. Curr. Genet. 27, 184-189.

Newton, K. J. (1988). Plant mitochondrial genomes: organization, expression, and variation. Ann. Rev. Plant Physiol. Plant Mol. Biol. 39, 503-532.

Nivison, H. T., Sutton, C. A., Wilson, R. K., and Hanson, M. R. (1994). Sequencing, processing, and localization of the petunia CMS-associated mitochondrial protein. The Plant J. 5, 613-623.

Nugent, J. M., and Palmer, J. D. (1991). RNA-mediated transfer of the gene *coxII* from the mitochondrion to the nucleus during flowering plant evolution. Cell 66, 473-481.

Oldenburg, D. J., and Bendich, A. J. (1996). Size and structure of replicating mitochondrial DNA in cultured tobacco cells. The Plant Cell 8, 447-461.

Op den Camp, R. G. L., and Kuhlemeier, C. (1997). Aldehyde dehydrogenase in tobacco pollen. Plant Molecular Biology 35, 355-365.

Ozias-Akins, P., Tabaeizadeh, Z., Pring, D. R., and Vasil, I. K. 1988. Preferential amplification of mitochondrial DNA fragments in somatic hybrids of the *Gramineae*. Curr. Genet. 13, 241-245.

Palmer , J. D., and Hebron, L. A. (1986). Tricircular mitochondrial genomes of *Brassica* and *Raphanus*: reversal of repeat configuration by inversion. Nucleic Acids Res. 14, 9755-65.

Palmer , J. D., and Hebron, L. A. (1987). Unicircular structure of the *Brassica hirta* mitochondrial genome. Curr. Genet. 11, 565-70.

Palmer, J. D., and Shields, C. R. (1984). Tripartite structure of the *Brassica campestris* mitochondrial genome. Nature 307, 437-440.

Perrotta, G., Malek, O., Heiser, V. Brennicke, A., Grohmann, L., and Quagliariello, C. (1996). RNA editing in the *cox3* mRNA of *Magnolia* is more extensive than in other dicot or monocot plants. Biochimica et Biophysica Acta 1307, 254-258.

Phreaner, C. G., Williams, M. W., and Mulligan, R. M. (1996). Incomplete editing of *rps12* transcripts results in the synthesis of polymorphic polypeptides in plant mitochondria. Plant Cell 8, 107-117.

Pring, D. R., and Lonsdale, D. M. (1985). Molecular biology of higher plant mitochondrial DNA. International Rev. of Cytology 97, 1-41.

Pring, D., Brennicke, A., and Schuster, W. (1993). RNA editing gives a new meaning to the genetic information in mitochondria and chloroplasts. Plant Mol. Bio. 21, 1163-1170.

Rajasekhar V. K., and Mulligan, R. M. (1993). RNA editing in plant mitochondria: a-phosphate is retained during C-to-U conversion in mRNAs. The Plant Cell 5, 1843-1852.

Rapp, W. D., and Stern, D. B. (1992). A conserved eleven nucleotide sequence contains an essential promoter element of the maize mitochondrial *atp1* gene. EMBO J. 11, 1065-73.

Rapp, W. D., Lupold, D. S., Mack, S., and Stern, D. B. (1993). Architecture of the maize mitochondrial *atp1*promoter as determined by linker-scanning and point mutagenesis. Mol. Cell. Biol. 13, 7232-7238.

Rhodes, M. M. (1950). Gene induced mutation of a heritable cytoplasmic factor producing male sterility in maize. Proc. Natl. Acad. Sci. USA 36, 634-635.

Rottmann, W. H., Brears, T., Hodge, T. P., and Lonsdale, D. M. (1987). A mitochondrial gene is lost via homologous recombination during reversion of CMS T maize to fertility. EMBO J. 6, 1541-1546.

Sakamoto, W., Kondo, H., Murata, M., and Motoyoshi, F. (1996). Altered mitochondrial gene expression in a maternal distorted leaf mutant of *Arabidopsis* induced by *chloroplast mutator.* Plant Cell 8, 1377-1390.

Salazar, R. A., Pring, D. R., and Kempken, F. (1991). Editing of mitochondrial *atp9* transcripts from two sorghum lines. Curr. Genet. 20, 483-486.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain termination inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463-5467.

Schuste, W., Ternes, R., Knoop, V., Hiesel, R., Wissinger, B., and Brennicke, A. (1991). Distribution of RNA editing sites in *Oenothera* mitochondrial mRNAs and rRNAs. Curr. Genet. 20, 397-404.

Schuster, W., and Brennicke, A. (1991). RNA editing in *ATPase* subunit 6 mRNAs in *Oenothera* mitochondria: a new termination codon shortens the reading frame by 35 amino acids. FEBS Lett. 295, 97-101.

Schuster, W., and Brennicke, A. (1994). The plant mitochondrial genome: physical structure, information content, RNA editing, and gene migration to the nucleus. Ann. Rev. Plant Physiol. Plant Mol. Biol. 45, 61-78.

Senda, M., Mikami, T., and Kinoshita, T. (1993). The sugar beet mitochondrial gene for the ATPase alpha-subunit: sequence, transcription and rearrangements in cytoplasmic male-sterile plants. Curr. Genet. 24, 164-170.

Shadel, G. S., and Clayton, D. A. (1997). Mitochondrial DNA maintenance in vertebrates. Annu. Rev. Biochem. 66, 409-435.

Singh, M. and Brown, G. G. (1991). Suppression of cytoplasmic male sterility by nuclear genes alters expression of a novel mitochondrial gene region. Plant Cell3, 1349- 1362.

Small, I. D., Isaac, P. G., and Lever, C. J. (1987). Stoichiometric differences in DNA molecules containing the *atpA* gene suggest mechanisms for the generation of mitochondrial genome diversity in maize. EMBO J. 6, 865-869.

Small, I. D., Suffolk, R., and Leaver, C. J. (1989). Eevolution of plant mitochondrial genomes via substoichiometric intermediates. Cell 58, 69-75.

Smart, C. J., Moneger, F., and Leaver, C. J. (1994). Cell-specific regulation of gene expression in mitochondria during anther development in sunflower. The Plant Cell 6, 811-825.

Song, J., and Hedgcoth, C. (1994). Influence of nuclear background on transcription of a chimeric gene (*orf256*) and *coxI* in fertile and cytoplasmic male sterile wheats. Genome 37, 203-209.

Stahl, R., Sun, S., L'Homme, Y., Ketela, T., and Brown, G. G. (1994). RNA editing of transcripts of a chimeric mitochondrial gene associated with cytoplasmic male-sterility in *Brassica*. Nucleic Acid Res. 22, 2109-2113.

Stern, D. B. and Palmer, J. D. (1984). Extensive and widespread homologes between mitochondrial DNA and chloroplast DNA in plants. Proc. Natl. Acad. Sci. USA 81, 1946-1950.

Suyama, Y., and Bonner, W. D. (1966). DNA from plant mitochondria. Plant Physiol. 41, 383-388.

Tadege, M., and Kuhlemeier, C. (1997). Aerobic fermentation during tobacco pollen development. Plant Molecular Biology 35, 343-354.

Tang, H. V., Pring, D. R., Shaw, L. C., Salazar, R. A., Muza, F. R., and Schertz, K. F. (1996). Transcript processing internal to a mitochondrial open reading frame is correlated with fertility restoration in male sterile sorghum. The Plant J. 10, 132-133.

Thomson, M. C., Macfarlane, J. L., Beagley, C. T., and Wolstenholme, D. R. (1994). RNA editing of mat-r transcripts in maize and soybean increase similarity of the encoded protein to fungal and bryophyte group II intron maturases: evidence that mat-r encodes a functional protein. Nucleic Acids Res. 22, 5745-5752.

Tzagoloff, A. (1982). *Mitochondria.* Plenum Press, New York.

Unseld, M., Marienfeld, J. R., Brandt, P., and Brennicke, A. (1997). The mitochondrial genome of *Arabidopsis thaliana* contains 57 genes in 366,924 nucleotides. Nature Genetics 15, 57-61.

Warmke, H. E., and Lee, S-L. J. (1977). Mitochondrial degeneration in Texas cytoplasmic male-sterile corn anthers. J. Hered. 68, 213-222.

Ward, B. L., Anderson, R. S., and Bendich, A. J. (1981). The mitochondrial genome is large and variable in a family of plants (*Cucurbitaceae*). Cell 25, 793-803.

Ward, G. C., and Levings III, C. S. (1991). The Protein-encoding gene T-*urf13* is not edited in maize mitochondria. Plant Mol. Biol. 17, 1083-1088.

Wen, L-Y, and Chase, C. D. (1998). Pleiotropic effects of a nuclear restorer-of-fertility on mitochondrial gene expression in developing male gametophytes of CMS-S maize. In press.

Wilson, R. K., and Hanson, M. R. (1996). Preferential RNA editing at specific sites within transcripts of two plant mitochondrial genes does not depend on transcriptional context or nuclear genotype. Curr. Genet. 30, 502-508.

Wilson, A. J., Lloyed, C. K., Ragland, C. K., Chourey, P. S., and Chase, C. D. (1989). Unique properties of a 136 kb circular DNA associated with *Sorghum* mitochondria. Plant Sci. 61, 81-90.

Wissinger, B., Schuster, W., and Brennicke, A. (1991). Trans splicing in *Oenothera* mitochondria: nad1 mRNAs are edited in exon and *trans*-splicing group II intron sequences. Cell 65, 473-482.

Yan, B., and Pring, D. R. (1997). Transcriptional initiation sites in sorghum mitochondrial DNA indicate conserved and variable features. Curr. Genet. 32, 287-295.

Young, E. G., and Hanson, M. R. (1991) A fused mitochondrial gene associated with cytoplasmic male sterility is developmentally regulated. Cell 50, 41-49.

Yu, W., and Schuster, W. (1995). Evidence for a site-specific cytidine deamination reaction involved in C to U RNA editing of plant mitochondria. J. of Biol. Chem. 270, 18227-18233.

Zabala, G., Gabay-Laughnan, S., and Laughnan, J. R. (1997). The nuclear gene *Rf3* affects the expression of the mitochondrial chimeric sequence R implicated in S-type male sterility in maize. Genetics 147, 847-860.

Zanlungo, S., Begu, D., Quinones, V., Araya, A., and Jordana, X. (1993). RNA editing of apocytochrome b (*cob*) transcripts in mitochondria from two genera of plants. Curr. Genet. 24, 344-348.

BIOGRAPHICAL SKETCH

Byoung Ok Kim was born in the Republic of Korea on the first day of September, 1959. Young Geun Kim and Seoung Soon Oh, my parents, spent their whole lives for me and family. My youth was great because I did not know anything about responsibility and right. I went to the University of Kon Kook in 1978 to study advanced biotechnology for agriculture. Three years of experience in the Agricultural Development Corporation were invaluable to change my view point of the real, rural world and led me to fly to the U. S. A. to the challenge of an unsecured future-not just for bread, but for the things to which I can devote my life. North Dakota State University was the place where I earned my M. S. degree in the U. S. A. All I can think of North Dakota is freezing cold and windy snow. From March 1988 to August 1990, I studied overall methylation changes during development of the potato, ND-860. I still have the methylation ghost in my mind and hope to study further the mechanism of the methylation. I came to Gainesville 1992 to join the Chase Lab to study CMS in common bean.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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